University of Alberta

# M11L: An anti-apoptotic protein of the Myxoma poxvirus

By

Helen Everett ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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## Abstract

M11L, a novel 166aa membrane-associated protein expressed by the poxvirus, myxoma virus, was previously found to modulate apoptosis following infection of rabbit leukocytes. Furthermore, infection of rabbits with an M11L knockout virus unexpectedly produced lesions with a profound pro-inflammatory phenotype. The current study shows that M11L is anti-apoptotic when expressed independently from other viral proteins and acts downstream of Bid cleavage and upstream of cytochrome c release. M11L is directed specifically to mitochondria by a short C-terminal region that is necessary and sufficient for targeting. This targeting region consists of a hydrophobic domain flanked by basic amino acid residues, adjacent to a positively charged tail. M11L blocks staurosporine-induced apoptosis by preventing mitochondria from undergoing a permeability transition and membrane association of this protein is essential for this function. The R164G variant of M11L localizes to intracellular membranes including the endoplasmic reticulum, rather than to mitochondria and has reduced anti-apoptotic properties. M11L also prevents apoptosis induced by PK11195 and protoporphyrin IX, two ligands of the mitochondrial peripheral benzodiazepine receptor (PBR) component of the permeability transition pore. Crosslinking studies indicate that M11L physically associates with the PBR. In addition, myxoma virus infection and the associated expression of early proteins,

including M11L, protects cells from staurosporine-mediated mitochondrial membrane potential loss and this effect is augmented by the presence of PBR. Taken together, these findings suggest that M11L regulates the mitochondrial permeability transition pore complex, most likely by direct modulation of the PBR. M11L is specifically required to inhibit the apoptotic response of monocytes/macrophages during virus infection as cells of this lineage undergo apoptosis when infected with the M11L knockout virus. As monocyte apoptosis is uniquely pro-inflammatory, this observation reconciles the paradoxical pro-apoptotic and pro-inflammatory phenotypes of the M11L knockout virus. This finding suggests that apoptosis of tissue macrophages represents an important anti-viral defense, and that the inhibition of apoptosis by viral proteins can be directed in a cell-specific fashion.

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## List of non-standard abbreviations

| | |
|---|---|
| $\Delta\Psi_m$ | mitochondrial membrane potential |
| ADP | adenovirus death protein |
| AIF | apoptosis inducing factor |
| ANT | adenine nucleotide transporter |
| BH | Bcl homology |
| CARD | caspase recruitment domain |
| CAD | caspase-activated DNase |
| CCCP | carbonyl cyanide m-chlorophenyl hydrazone |
| CK | creatine kinase |
| COX IV | cytochrome c oxidase subunit IV |
| CPD | cyclophilin D |
| DD | death domain |
| DED | death effector domain |
| $DiOC_6$ | 3,3'dihexyloxacarbocyanine iodide |
| DSS | disuccinimidyl suberate |
| DSP | dithiobis(succinimidylpropionate) |
| ECL | enhanced chemiluminescence |
| EGFP | Enhanced Green Fluorescent Protein |
| HK | hexokinase |
| IAPs | inhibitor of apoptosis proteins |
| HBx | hepatitis B virus protein x |
| ICAD | inhibitor caspase-activated DNase |
| ICE | Interleukin 1β converting enzyme (caspase-8) |
| MOI | multiplicity of infection |
| PARP | poly(ADP-ribose) polymerase |
| PBR | peripheral benzodiazepine receptor |
| PE | phycoerythrin |
| PKR | protein kinase, dsRNA activated |
| PT | permeability transition |
| PPIX | protoporphyrin IX |

| | |
|---|---|
| PT | permeability transition |
| STS | staurosporine |
| TMRE | ethyl ester of tetramethylrhodamine |
| TNF(R) | tumor necrosis factor (receptor) |
| TRADD | tumor necrosis factor receptor-associated death domain protein |
| TRAF | tumor necrosis factor associated factor |
| TRAIL | TNF-related apoptosis-inducing ligand |
| TUNEL | terminal deoxynucleotidyl transferase-(TdT) mediated dUTP-fluorescein nick end labeling |
| VDAC | voltage dependent anion carrier |
| vFLIPs | viral FLICE (caspase-8) inhibitory proteins |
| vMIA | viral mitochondrial inhibitor of apoptosis |
| Vpr | viral protein R |

## List of Virus names

| | |
|---|---|
| BHV | Bovine Herpesvirus |
| EBV | Epstein-Barr virus |
| HCMV | Human cytomegalovirus |
| HBV | Hepatitis B virus |
| HCMV | Human cytomegalovirus |
| HCV | Hepatitis C virus |
| HSV | Herpes simplex virus |
| HIV | Human immunodeficiency virus |
| HPV | Human Papillomavirus |
| HTLV | Human T cell leukemia virus |
| HVS | Herpesvirus saimiri |
| HHV-8 | Human herpervirus 8 |
| KSV | Kaposi's sarcoma-associated virus |
| MCV | Molluscum contagiosum virus |

| | |
|---|---|
| VV601 | Vaccinia virus |
| VVM11L | M11L-expressing vaccinia virus |
| vMyxlac | Myxoma virus |
| vMyxM11L⁻ | M11L knockout myxoma virus |

# Chapter 1

## Introduction[1]

[1] Sections of this introduction have been previously published (Everett and McFadden, 1999; Everett and McFadden, 2001; Everett and McFadden, 2001)

## Intersecting influences: cell death pathways and virus infection

Apoptosis is a physiological process of cell death whereby individual cells within metazoan organisms can be eliminated. This form of cell death plays a central role in many processes fundamental to life, such as development, homeostasis and disease prevention (Hengartner, 2000; Rich et al., 1999). In particular, the potential for apoptosis to serve as a specific mechanism for the removal of virus-infected cells from multicellular organisms was recognized historically (Clouston and Kerr, 1985) and continues to be supported by accumulating experimental evidence. Indeed, apoptosis is so effective in curtailing a wide range of virus infections that this cell death process might even be considered a component of host innate immunity (Everett and McFadden, 1999; O'Brien, 1998; Roulston et al., 1999). Nevertheless, viruses continue to be maintained within permissive host populations, indicating the existence of a dynamic equilibrium between the conflicting influences of the need for the virus to replicate and the need for the host to resist infection.

In most cases, it seems that the induction of apoptosis is an unavoidable result of virus entry and infection. Apoptosis of infected cells may be triggered by virus-specific factors or as a result of recognition by cytotoxic T lymphocytes or natural killer cells (reviewed in (Edwards et al., 1999; Roulston et al., 1999; White, 1998)). Consequently, many viruses have developed highly efficient strategies to curtail the host's protective apoptotic response to infection. This is particularly apparent with viruses that have long replication cycles, such as members of the adenovirus, lymphotrophic herpesvirus and poxvirus families (O'Brien, 1998; Roulston et al., 1999; Tschopp et al., 1998). The evolution of these viral strategies is thought to confer an advantage in terms of the ability of viruses to replicate, disseminate and/or establish persistent infections. Alternatively,

the virus-induced apoptotic response may be limited with respect to the numbers or lineages of cells infected and virus replication may be sufficient to allow successful transmission. This situation is observed, for example, during alphaherpesvirus or influenza virus infection (Balachandran et al., 2000; Keiff and Shenk, 1998). Conversely, other viruses have evolved to exploit the normally protective apoptotic response of the host. For example, when Sindbis virus infects neurons in newborn mice, completion of the viral replication cycle coincides with the onset of apoptotic body formation (Griffin and Hardwick, 1999; Lewis et al., 1996). Virus particles can then be transported within apoptotic bodies and taken up by phagocytic cells without exposure to other elements of the immune system in the extracellular milieu. Similarly, at late stages of adenovirus infection, the adenovirus death protein (ADP) is produced. This protein induces a form of atypical 'programmed cell necrosis' that facilitates release of progeny virions and increases virus yield (Tollefson et al., 1996).

This chapter will introduce the subject of apoptosis and viral strategies that are employed to modulate this cell death process, with particular emphasis on key regulatory events involving mitochondria. Finally, apoptosis modulation by the myxoma poxvirus, and particularly by the M11L protein, will be discussed.

## Apoptotic cell death

Apoptosis was first identified as distinct form of cell death on the basis of characteristic morphological criteria (Kerr et al., 1972; Wyllie, 1980). The morphological changes that accompany death by apoptosis include cell shrinkage, chromatin condensation and the formation of plasma membrane blebs prior to partitioning of the cellular contents into membrane-enclosed apoptotic bodies. *In vivo*, these apoptotic bodies are

then engulfed by surrounding phagocytes with minimal impact on surrounding tissues. The contrasting process of necrosis involves cellular swelling and lysis with the release of the cell contents into the extracellular milieu. Necrosis within tissues can therefore initiate a severe inflammatory response (Haanen and Vermes, 1995). The physical changes that are observed during apoptosis are linked to defined molecular events primarily involving two classes of proteins: the caspases and the Bcl-2 family members.

**Caspases**

Caspases or cysteinyl aspartate-specific proteinases play an essential role during apoptotic cell death and are included in a new class of cysteine protease that comprise a multi-gene family (reviewed in (Budihardjo et al., 1999; Earnshaw et al., 1999; Nicholson, 1999)). Caspases are characterized by their absolute requirement for hydrolysis of peptide bonds on the carboxyl side of aspartate residues. The aspartate residue adjacent to each cleavage site (designated the P1 residue) together with the three substrate residues that are immediately N-terminal (designated P2, P3 and P4 progressing towards the N-terminus) form the substrate recognition sequence that defines the specificity of each caspase (Garcia-Calvo et al., 1998; Thornberry et al., 1997). The only other known protease with a similar preference for cleavage after aspartate resides is the serine protease granzyme B, itself a protease that is able to activate apoptotic cascades upon release into target cells from activated cytotoxic T cell granules (Andrade et al., 1998; Darmon et al., 1995; Martin et al., 1996).

Caspases can be divided into three subgroups based on their substrate specificity and activity (Figure 1.1). The group I caspases are primarily involved in cytokine processing and inflammation and require a hydrophobic residue in the P4 position of the substrate. ICE (interleukin

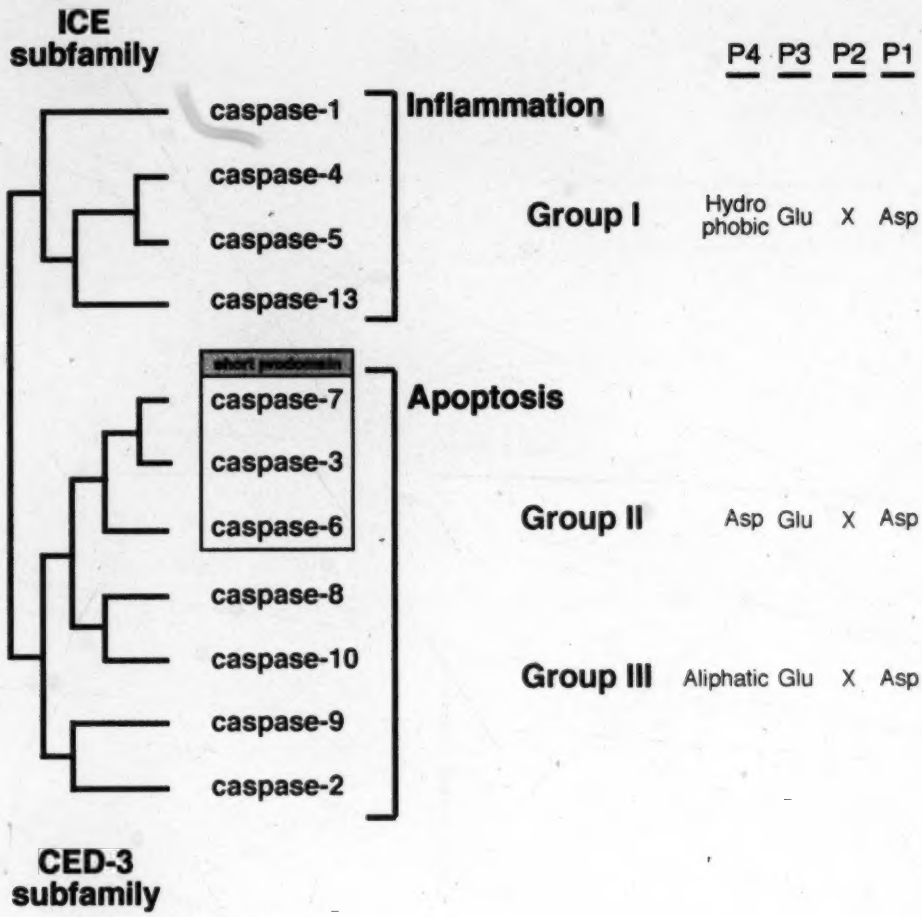


**Figure 1.1 The Caspase gene family.** The caspases can be involved in inflammatory cytokine processing (Group I) or apoptosis (Groups II and III). The caspase groups are distinguished according to phylogenetic category (ICE or CED-3 subfamily) and specificity for the residues in positions P1 to 4 in the substrate cleavage site. All caspases cleave C-terminal to an aspartate residue in the P1 position. Adapted from (Nicholson, 1999).

1β-converting enzyme; caspase-1) is the prototypal member of this group. Group II and III caspases are involved in apoptosis and demonstrate a requirement for aspartate or an aliphatic residue at the P4 position in the substrate recognition site respectively (Nicholson, 1999). Group II and III caspases are also distinct with regard to their structural properties and modes of activation. Caspases are present in cells as inactive zymogens or procaspases and undergo activation by proteolytic processing. Each precursor molecule undergoes cleavage to separate a large and small subunit and then remove an N-terminal regulatory prodomain. The large and small subunits from two molecules then assemble to form the active caspase.

The group III caspases are upstream activators of cell death and are often termed initiator caspases. These caspases have large pro-domains and become activated by a process of 'scaffold mediated transactivation' (Earnshaw et al., 1999). According to this process, procaspases are recruited into a complex that assembles in response to a death signal. This increase in the local concentration of caspase molecules and/or a conformational change enhances a low endogenous activity. This mechanism of activation has been well characterized with respect to two initiator caspases, namely caspase-8 and –9.

Caspase-8 is involved in cell death initiated by cell surface death receptors. Following binding of a death receptor, such as Fas or the tumor necrosis factor receptor (TNFR) for example, to a cognate ligand (such as Fas L or tumor necrosis factor (TNF), respectively), ligand-induced receptor trimerization leads to the assembly of a cytoplasmic molecular complex. A key component of this complex is FADD, an adaptor molecule that contains two death-effector domain (DED) motifs that are capable of homotypic interactions (Boldin et al., 1995; Chinnaiyan et al., 1995; Hsu et al., 1995; Muzio et al., 1996). The prodomain of procaspase-8 also contains

a DED motif, and this facilitates oligomerization and trans-activation of caspase molecules in the same scaffold structure, leading to full proteolytic activity (Martin et al., 1998; Muzio et al., 1998; Yang et al., 1998).

Caspase-9 is the primary caspase that is activated downstream of the mitochondrial cell death control point (discussed later in this Chapter). Procaspase-9 is activated following interaction with a scaffolding molecule, Apaf-1, by means of homotypic caspase-recruitment (CARD) domains (Hofmann et al., 1997; Hu et al., 1998). However, procaspase-9 activation only occurs if Apaf-1 is rendered functional by prior binding and hydrolysis of ATP or dATP and incorporation of mitochondria-derived holocytochrome c (Li et al., 1997; Liu et al., 1996). Pro-caspase-9 is recruited to the Apaf-1/cytochrome c complex in 1:1 stoichoimetry, suggesting that Apaf-1-mediated procaspase-9 activation occurs by means of autocatalysis (Zou et al., 1999).

The group III initiator caspases have as their major substrates pro-apoptotic Bcl-2 family members and the effector caspases. The effector (group II) caspases are therefore activated as a result of cleavage by initiator caspases at aspartate-containing cleavage sites. The effector caspases have short prodomains and are typified by caspase-3, the predominant effector caspase that is activated once a cell is irreversibly committed to apoptosis. Caspase-3 and the other effector caspases orchestrate many of the morphological events characteristic of apoptosis by bringing about the proteolytic cleavage of a diversity of cytoplasmic and nuclear substrates involved in the maintenance of cellular homeostasis and structure (Earnshaw et al., 1999; Nicholson, 1999).

Effector caspase substrates in the cytoplasm are abundant and include signal transduction factors (e.g. a large number of protein kinases) and

structural proteins (e.g gelsolin, fodrin and actin). Nuclear caspase substrates encompass proteins involved in regulation of proliferation and the cell cycle (e.g. $p21^{Cip1/Waf1}$ and pRB), nuclear structural proteins (e.g the nuclear lamins A and B) and DNA metabolism and repair proteins (e.g. poly-(ADP ribose) polymerase and DNA topoisomerase II). Effector caspases, in particular caspase-3, also initiate DNA fragmentation by activating the caspase-activated DNase (CAD). Specifically effector caspases cleave and inactivate the inhibitor of this enzyme, ICAD, thereby liberating CAD that then brings about the cleavage of DNA at internucleosomal regions that is a hallmark of apoptosis (Enari et al., 1998; Liu et al., 1997; Sakahira et al., 1998; Tang and Kidd, 1998). Caspase-3 is also responsible for activating other effector caspases and completing an amplification loop by activating residual unprocessed initiator caspase molecules. The cumulative effect of caspase activation, therefore, is disruption of the maintenance, repair and anti-apoptotic pathways within the cell and dismantling of sturctural components. The final outcome of this process is the packaging of the cellular contents into membrane-bound apoptotic bodies, which display exposed phosphatidyl serine on their surface and are therefore marked for removal by phagocytosis.

**The Bcl-2 family of proteins**

Bcl-2 proteins play key regulatory roles in cell death and are identified by the presence of between one and four small α-helical segments designated Bcl-2 homology (BH) domains (reviewed in (Gross et al., 1999; Harris and Thompson, 2000; Vander Heiden and Thompson, 1999)). The BH domains are important for the function of both the anti-apoptotic Bcl-2 family members such as Bcl-2 and Bcl-$x_L$ and the pro-apoptotic family members such as Bid, Bax and Bak (Figure 1.2). NMR structural analysis of Bcl-$x_L$ has revealed that the BH 1, 2 and 3 domains form an elongated hydrophobic cleft (Muchmore et al., 1996) that can bind the amphipathic

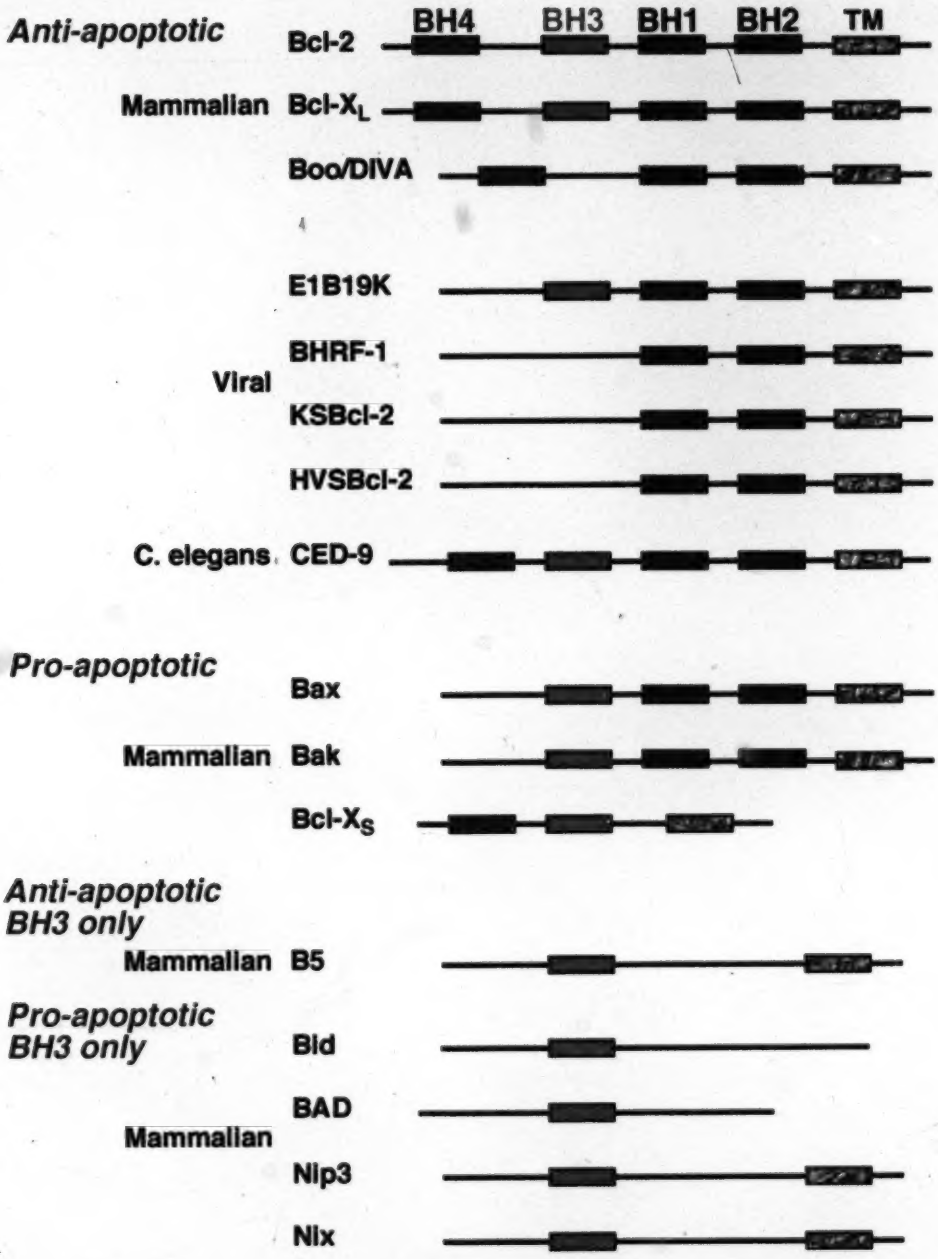


**Figure 1.2 The Bcl-2 family of proteins.** Anti- and Pro-apoptotic Bcl-2 proteins are characterized by the presence of Bcl homology (BH) domains and may or may not contain a transmembrane (TM) region. (Adapted from Gross, et al., 1999)

BH3 α-helices of the death-promoting protein Bak (Sattler et al., 1997) . The importance of BH3 domains for the function of death agonists is emphasized by the fact that a number of these molecules, notably Bid, and Bad, only contain this domain. BH4 domains are mainly restricted to anti-apoptotic proteins, with this domain in Bcl-2/Bcl-$x_L$ being involved in pro-survival functions by binding to death regulatory proteins e.g. Raf-1, and calcineurin (Reed, 1997).

Bcl-2 protein function is well controlled, although not always well understood. The regulation of Bcl-2 proteins was initially proposed to occur as a result of their ability to form homo- and heterodimers (Diaz, 1997; Oltavi et al., 1993). Bcl-2 or Bax homodimers were attributed strongly anti- or pro-apoptotic properties respectively. Formation of heterodimers between anti- and pro-apoptotic proteins was thought to be responsible for titrating their opposing activities, with the effects of the proteins present in highest concentration being predominant (Oltvai and Korsmeyer, 1994). This model is supported by several experimental findings. Specifically, site-directed mutagenesis approaches have revealed a correlation between the ability of Bcl-2 proteins to heterodimerize and their ability to regulate apoptosis (Chang, 1999; Zha et al., 1997). In addition, small molecule inhibitors that prevent heterodimerization mediated by the BH3 domains of pro-apoptotic Bcl-2 proteins are pro-apoptotic, presumably because they prevent interaction between pro- and anti-apoptotic family members (Cosulich et al., 1997; Degterev et al., 2001; Holinger et al., 1999).

Recently, however, the heterodimerization model has been called into question when it was found that Bcl-$X_L$ mutants, that do not bind Bax or Bak, are nevertheless able to inhibit cell death (Cheng et al., 1996). In addition, a Bcl-2 protein, KSBcl-2, expressed by human herpesvirus 8, although functionally anti-apoptotic, does not form homo- or

heterodimers (Cheng et al., 1997). Therefore, although heterodimer formation does seem to play some role in the function of Bcl-2 proteins, additional intrinsic properties, in particular the ability of these proteins to influence mitochondrial processes, are also important (Minn et al., 1999).

To date, the post-translational modifications that have been identified as significant for the regulation of Bcl-2 proteins include phosphorylation, cleavage and conformational changes. Frequently, these modifications are also accompanied by an alteration in the intracellular localization of the protein in question. Phosphorylation is particularly important for the regulation of the 'BH3 only' pro-apoptotic protein BAD. When phosphorylated on specific serine residues ($ser^{112}$ or $ser^{136}$), BAD is sequestered by 14-3-3 proteins in the cytoplasm and rendered inactive (Zha et al., 1996). However, during the induction of apoptosis, BAD can become dephosphorylated exposing the BH3 domain and allowing heterodimer formation with Bcl-2/Bcl-$X_L$, redistribution to mitochondria and the promotion of cell death (Zha et al., 1997). Interestingly, phosphorylation on $ser^{155}$ within the BH3 domain renders BAD incapable if interaction with Bcl-2 and unable to induce apoptosis (Zhou et al., 2000). Bcl-2 and Bik are also known to undergo phosphorylation, a modification that influences the normal function of these proteins (Chang et al., 1997; Haldar et al., 1995; Ito et al., 1997; Verma et al., 2001).

Both anti- and pro-apoptotic Bcl-2 proteins are known to be regulated by cleavage. Bcl-2/Bcl-$X_L$ can be cleaved by caspases to produce C-terminal cleavage products that are highly pro-apoptotic (Cheng et al., 1997; Clem et al., 1998) and Bax cleavage accentuates its pro-apoptotic activity (Wood et al., 1998). These effects perhaps reflect enhanced exposure of the BH 3 domains of these molecules. A similar cleavage mechanism is also essential for the activation of the pro-apoptotic, 'BH3 only' molecule, Bid. Bid plays a role in amplifying many apoptotic cascades when, upon

initiation of a death signal, this protein undergoes caspase-mediated cleavage to liberate a 15 kDa C-terminal fragment (tBid) (Li et al., 1998; Luo et al., 1998). A significant aspect of Bid signaling is the redistribution of this molecule to the outer mitochondrial membrane and concomitant recruitment of Bax or Bak to the same location (Desagher et al., 1999; Wei, 2000). The redistribution of Bax is associated with conformational changes that promote oligomerization and integration into the mitochondrial outer membrane (Goping et al., 1998; Gross et al., 1998; Nechushtan et al., 1999; Suzuki et al., 2000), a process that can be countered by Bcl-2 (Antonsson et al., 2001; Mikhailov et al., 2001). A similar mechanism is also thought to apply in the case of Bak activation (Desagher et al., 1999; Griffiths et al., 1999; Wei et al., 2001). Interestingly, the formation of tBid exposes a hydrophobic region containing the BH3 domain (Chou et al., 1999), and also unmasks an N-terminal myristoylation site (Zha et al., 2000). This increase in hydrophobicity is thought to be the driving force for integration into the mitochondrial outer membrane.

The precise biochemical mode of action of the Bcl-2 proteins is not defined, however a common theme is that their site of action is largely localized at the outer mitochondrial membrane where they appear to contribute or modulate channel activity (reviewed in (Gross et al., 1999; Vander Heiden and Thompson, 1999)). Bcl-2 itself is present in the outer mitochondrial membranes as well as the perinuclear and endoplasmic reticulum membranes, a sub-cellular distribution that was confirmed using diverse approaches including immunelectron microscopy, subcellular fractionation and confocal microscopy (de Jong et al., 1994; Jacobson et al., 1993; Krajewski et al., 1993; Monaghan et al., 1992). This membrane association is mediated by the C-terminal transmembrane domain (Chen-Levy and Cleary, 1990). Absence of this domain renders Bcl-2 non-functional, indicating that membrane association is required for

function (Nguyen et al., 1994; Tanaka et al., 1993). These observations have refocused attention on a third participant required for the effector phase of many apoptotic signals, namely mitochondrial organelles.

## Mitochondrial cell death pathways

Mitochondria seem to be particularly important for relaying and amplifying so-called 'intrinsic' cell death signals that emanate from within the cell. These signals are triggered by diverse stimuli including growth factor withdrawal, glucocorticoid treatment, effects of cytotoxic agents, protein kinase inactivation and translational arrest (reviewed in (Crompton, 2000; Desagher and Martinou, 2000; Kroemer and Reed, 2000)). A hallmark of intrinsic cell death is that caspases (such as caspase-9) are activated downstream of the mitochondrial checkpoint. In some cases, cell death is observed even in the presence of caspase inhibitors (Deas et al., 1998), suggesting that mitochondria can also orchestrate caspase-independent cell death. 'Extrinsic' death signals are transmitted into a cell from the exterior by, for example, ligation and trimerization of cell surface death receptors of the Fas/TNFR family. Each of these receptors contain, in their cytoplasmic domain, a motif known as a death domain (DD). Oligomerization of these DDs initiates assembly of a molecular platform by recruitment of DD-containing adaptor molecules through homotypic interactions. The Fas death receptor (Figure 1.3) recruits FADD to the complex (Boldin et al., 1995; Chinnaiyan et al., 1995), whereas activation of the TNF receptor results in the recruitment of another DD-containing adaptor molecule, TRADD, which then recruits FADD (Hsu et al., 1995).

FADD is responsible for propagating the signal by mediating the recruitment and activation of 'initiator' caspases such as caspase-8 as already described (Martin et al., 1998; Muzio et al., 1998; Yang et al., 1998).

In the prototypal Fas pathway (Figure 1.3), cells can be categorized into two types according to whether or not mitochondrial signaling is required (Scaffidi et al., 1998; Scaffidi et al., 1999). In so-called Type I cells, the apoptotic signal is sufficiently robust to bypass the mitochondrial amplification step and caspase-8 is able to directly activate effector caspases, such as caspase-3. In Type II cells, only modest activation of caspase-8 occurs (Scaffidi et al., 1998; Scaffidi et al., 1999). However within the classical Fas pathway, caspase activity is sufficient to activate Bid, resulting in the recruitment of Bid, Bax and/or Bak to mitochondria and relay the extrinsic death signal. A mitochondrial event then initiates release of apoptogenic proteins into the cytoplasm, activating caspase-9 which, in turn activates caspase-3, resulting in apoptosis (reviewed in (Hueber, 2000)).

**Mechanisms of mitochondrial cell death signaling**

Although mitochondria play a central role in many cell death pathways, the mechanistic details whereby these organelles contribute to the demise of a cell are still a matter of some debate (reviewed in (Kroemer and Reed, 2000; Martinou and Green, 2001; Zamzami and Kroemer, 2001)). Mitochondria are complex structures, consisting of an inner membrane that encloses the matrix and an outer membrane that encloses the intermembrane space. The inner membrane is characterized by numerous convoluted folds and contains the proteins required for the respiration-driven electron transport chain. As a result of electron transport, an electrochemical gradient is maintained across the inner mitochondrial membrane ($\Delta\Psi_m$) and protons are ejected into the intermembrane space to serve as the driving force for the generation of ATP by oxidative phosphorylation.

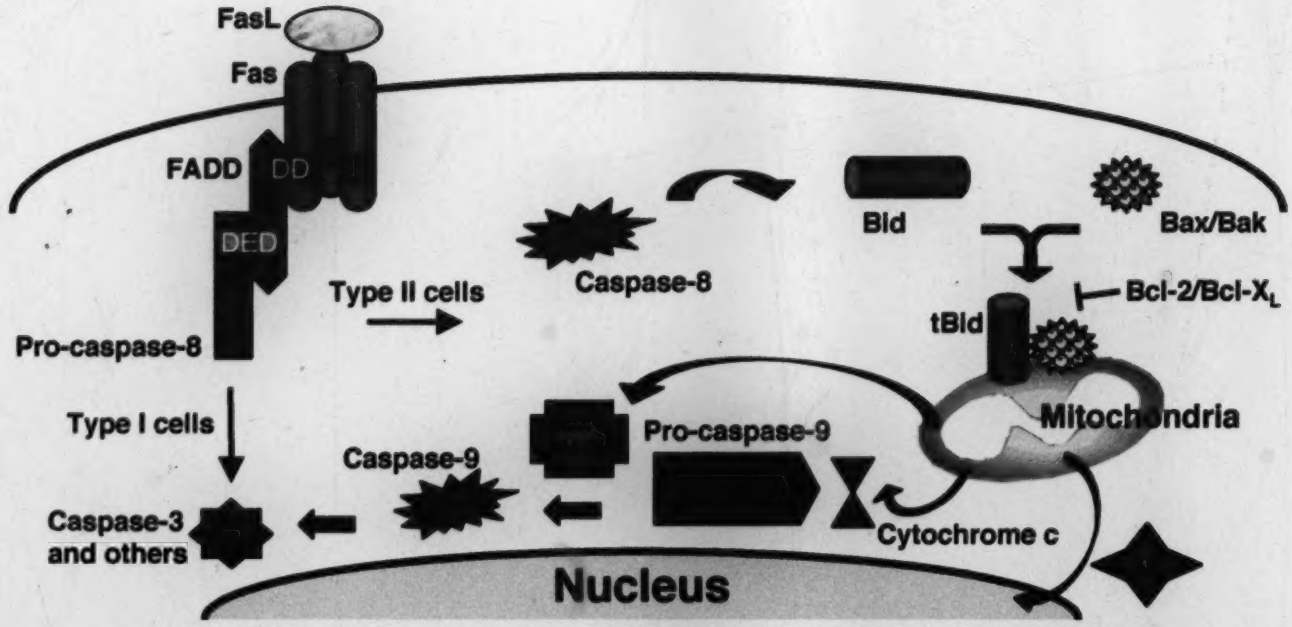


**Figure 1.3 The Fas apoptotic pathway.** Fas ligand (FasL) binds to the Fas death receptor inducing Fas trimerization and recruitment of the adaptor protein FADD by means of the Death Domain (DD). FADD, in turn, recruits pro-caspase-8 by means of the Death Effector Domain (DED) resulting in the release of cleaved, activated caspase-8. In Type I cells, caspase-8 activates caspase-3 directly. In Type II cells, the signal is amplified by the mitochondrial pathway. Caspase-8 cleaves and activates Bid which, in turn, recruits Bax and/or Bak to the mitochondrial outer membrane. This triggers release from mitochondria of apoptogenic proteins including Apoptosis inducing factor (AIF) and two proteins involved in pro-caspase-9 activation, namely Smac/Diablo and cytochrome c. Activated caspase-9 then activates the major effector caspase, caspase-3 that is mostly responsible for the morphological and biochemical effects of apoptosis.

Two characteristic changes in mitochondrial integrity can be observed during induction of an apoptotic response which involve marked perturbation of the outer or inner mitochondrial membrane respectively (reviewed in (Green and Reed, 1998; Kroemer and Reed, 2000; Martinou et al., 2000)). A consistent feature of apoptotic cell death seems to be the loss of outer membrane integrity allowing permeation of numerous proteins normally sequestered in the intermembrane space into the cytoplasm. Among the proteins released are mitochondrial pools of pro-caspases (such pro-caspases 2, 3 and 9), and the caspase activators cytochrome c and Diablo/Smac (Du et al., 2000; Liu et al., 1996; Verhagen et al., 2000). Cytochrome c and Diablo/Smac together assist in the cytoplasmic activation of pro-caspase-9 although it is unknown whether pro-caspase-9 molecules originating within the cytoplasm or mitochondria are involved. Another intermembrane space protein, Apoptosis Inducing Factor (AIF), also redistributes to the nucleus following an apoptotic signal. AIF is a flavoprotein and caspase-independent cell death effector that induces DNA fragmentation but operates via an unknown mechanism (Lorenzo et al., 1999; Susin et al., 1999).

Another mitochondrial event that accompanies apoptosis is the abrupt increase in the permeability of mitochondrial membranes that results in the dissipation of the electrochemical gradient across the inner membrane - an event known as the permeability transition (PT) (Zamzami et al., 1995). This is caused by the opening of the PT pore (Figure 1.4), a non-specific, multi-subunit pore or megachannel composed of the Voltage Dependent Anion Carrier (VDAC) that resides in the outer membrane, the Adenine nucleotide transporter (ANT) in the inner membrane and cyclophilin D that is associated with the matrix surface of the ANT (reviewed in(Crompton, 1999)). Pro- and anti-apoptotic Bcl-2 family members have been shown not only to regulate the PT pore but also to

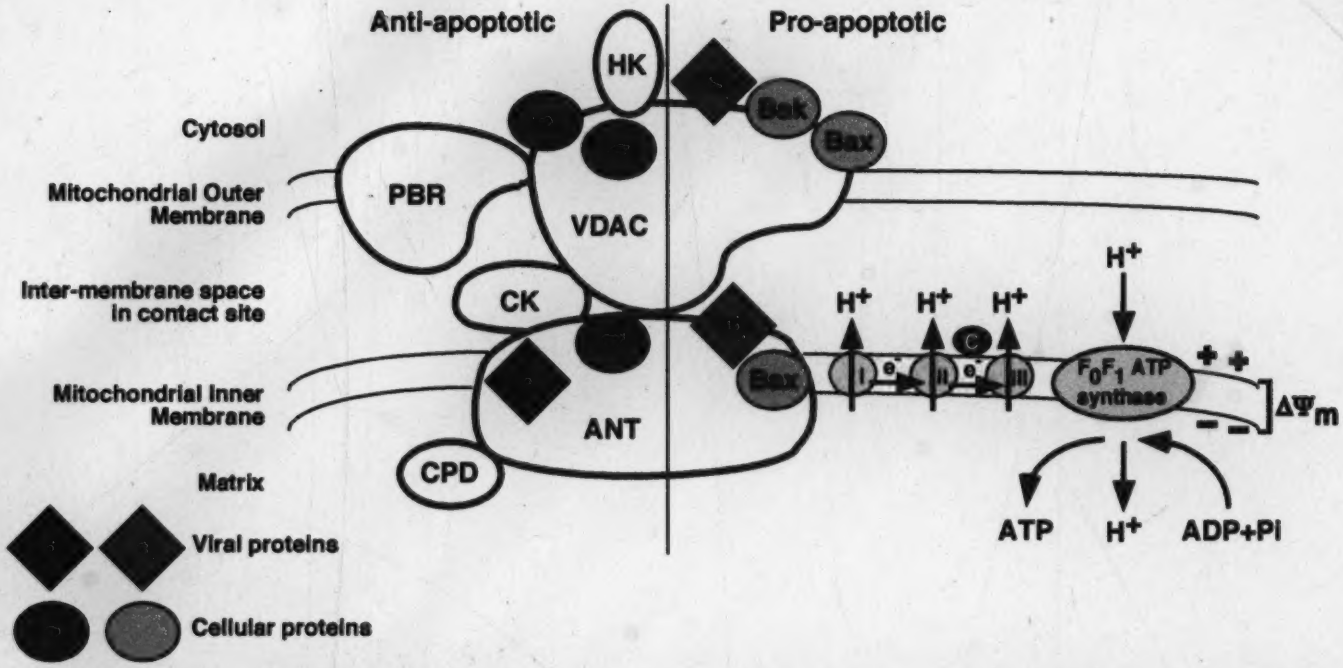


**Figure 1.4 The permeability transition (PT) pore.** The PT pore consists of the voltage dependent anion channel (VDAC) in the mitochondrial outer membrane, the adenine nucleotide translocator (ANT) in the mitochondrial inner membrane and cyclophilin D (CPD) in the mitochondrial matrix. Accessory proteins include hexokinase (HK) and the peripheral benzodiazepine receptor (PBR) associated with VDAC and creatine kinase (CK) associated with VDAC and ANT in the inter-membrane space. Several apoptotic modulators also associate with the PT pore subunits. These include the anti-apoptotic cellular proteins Bcl-2/Bcl-$X_L$ and viral protein vMIA as well as the pro-apoptotic cellular proteins Bax/Bak and viral proteins HBx and Vpr. The electron transport chain and associated cytochrome c (C) contributes to $\Delta\Psi m$ that drives the $F_0F_1$ ATP synthase and ATP generation.

physically associate with the VDAC and ANT components (reviewed in (Gross et al., 1999; Vander Heiden and Thompson, 1999)). The PT pore has other accessory proteins that may differ according to the cell type involved. These accessory proteins include Creatine Kinase associated with ANT-VDAC complex in the intermembrane space, Hexokinase II associated with the cytosolic face of VDAC and the Peripheral Benzodiazepine Receptor (PBR).

The PBR is an 18 kDa integral membrane protein, which resides primarily in the outer mitochondrial membrane (Anholt et al., 1986). Consistent with its inclusion in the PT pore, the PBR has been found to be both a physical and functional component of a complex containing VDAC and ANT (Kinnally et al., 1993; McEnery et al., 1992). PBR protein is concentrated in contact sites between the inner and outer mitochondrial membranes (Culty et al., 1999), as are PT pore assemblies and Bcl-2 (Dejong et al., 1994; Riparbelli et al., 1995; Zamzami and Kroemer, 2001). The PBR was first characterized as a benzodiazepine-interacting protein present in certain peripheral tissues peripheral to the central nervous system (Braestrup and Squires, 1977) and has since been shown to have a wide tissue distribution, being abundant in the adrenals, kidney, heart and reproductive tract, and also present in non-neuronal brain tissue (Gavish et al., 1999). Within leukocyte subsets, the highest concentrations of the PBR are found in monocytes and polymorphonuclear cells (Carayon et al., 1996; Dussossoy et al., 1996). The PBR shows highest affinity for the diazepine 4'-chlorodiazepam (Ro 5-4864) and the isoquinoline carboxamide analog, PK11195 (Le Fur et al., 1983; Marangos et al., 1982). The pharmacological properties and tissue distribution of the PBR distinguishes this receptor from the 'central type' benzodiazepine receptor that is present on the surface of cells of the central nervous system and preferentially binds the therapeutic diazepines (Zisterer and Williams, 1997). Numerous physiological functions have been proposed for the PBR,

including the ability to regulate cellular growth and proliferation, heme biosynthesis, cholesterol transport and steroidogenesis (Gavish et al., 1999) in addition to a role in the potentiation of inflammation (Klegeris et al., 2000; Torres et al., 2000; Torres et al., 1999; Waterfield et al., 1999) and apoptosis modulation (Carayon et al., 1996).

**The mitochondrial controversy: what is the death-defining event?**

Mitochondrial dysfunction could actively contribute to cell death by three mechanisms (Green and Reed, 1998). Firstly, perturbation in mitochondrial function could result in the lethal disruption of energy production. Secondly, the protective role of mitochondria in preserving redox balance would be removed. Thirdly, mitochondria appear to play an active part in the transduction cell death signals by, for example, releasing apoptogenic factors. The relative importance of the redistribution of mitochondrial proteins or disruption of mitochondrial function in the commitment of the cell to apoptosis remains controversial. There is evidence that the permeability transition and protein redistribution events are linked, although there have been reports of cytochrome c release occurring when $\Delta\Psi_m$ loss is transient, delayed or even absent. In other cases, however, transient $\Delta\Psi_m$ can precede limited cytochrome c release and does not result in apoptosis, provided caspases are not activated (reviewed in (Loeffler and Kroemer, 2000)). A recent report also indicates that mitochondrial alkalinization and cytosolic acidification can be detected before cytochrome c release and PT pore opening and this alteration in pH regulation constitutes the crucial determinant for apoptosis (Matsuyama et al., 2000). The precise function of the Bcl-2 proteins in regulating the mitochondrial control point is also not understood (Harris and Thompson, 2000).

**Models of mitochondria-mediated cell death**

Two models have been proposed to account for mitochondrial perturbations during cell death and also explain the role of the Bcl-2 family of proteins (reviewed in (Kroemer and Reed, 2000; Martinou et al., 2000)). The first model proposes that swelling of the mitochondrial matrix leads to osmotic rupture of the outer membrane, dissipation of $\Delta\Psi_m$ and release of apoptogenic factors. This model has been supported by ultrastructural studies (Feldman, 2000). Matrix swelling could result from an effect on ANT function causing formation of a non-specific pore in the mitochondrial inner membrane that allows influx of solutes and water into the matrix, thereby triggering an increase in matrix volume. In support of this, physical interactions between ANT and Bax, Bag-1, Bcl-2 and Bcl-$X_L$ have been reported (Brenner et al., 2000; Marzo et al., 1998; Marzo et al., 1998). Also, in artificial bilayers, Bax cooperates functionally with ANT to promote channel formation whereas Bcl-2 inhibits channel formation by ANT and can antagonize the channel opening properties of atractyloside, an ANT ligand in this system (Brenner et al., 2000; Marzo et al., 1998). Alternatively, matrix swelling could arise from VDAC-mediated selective permeabilization of the mitochondrial outer membrane. According to this model, VDAC closure following an apoptotic signal would lead to transient hyperpolarization of the inner membrane, leading to matrix swelling and outer membrane rupture. This model is supported by experimental data showing that, in some cell death processes, transient inner membrane hyperpolarization can occur (Vander Heiden et al., 1997). In addition, Bax and Bak were found to physically associate with VDAC present in isolated mitochondria and in this system cytochrome c release and $\Delta\Psi_m$ loss were either promoted or inhibited following addition of Bax/Bak or Bcl-2/Bcl-$X_L$ to mitochondria respectively (Narita et al., 1998). In a related study, Bcl-$X_L$ was shown to maintain metabolite

exchange across the outer mitochondrial membrane and prevent apoptosis by inhibiting VDAC closure (Vander Heiden et al., 2001).

The other model does not invoke outer membrane rupture, but instead proposes the formation of a regulated outer membrane pore to explain matrix protein translocation and $\Delta\Psi_m$ loss. Indeed, in some systems, mitochondrial disruption is not observed during apoptosis (Eskes et al., 2000; von Ahsen et al., 2000). Two means whereby mitochondrial pore formation might be accomplished have been suggested. The first proposes that, following induction of apoptosis, VDAC forms a selectively permeant pore (Shimizu et al., 1999). Such an occurrence is supported by studies using VDAC liposomes. In this system, Bax/Bak were able to potentiate cytochrome c passage through the VDAC pore whereas Bcl-2/Bcl-$X_L$ caused the pore to be cytochrome c impermeant (Shimizu et al., 1999). The functional region of Bcl-2/Bcl-$X_L$ required for this protective effect has been identified as the BH 4 domain. This domain alone was shown to be necessary and sufficient to prevent VDAC opening and $\Delta\Psi_m$ loss in isolated mitochondria and prevent apoptosis in intact cells (Shimizu et al., 2000). An alternative suggestion is that Bcl-2 proteins can form channels independently of the PT pore components. This theory was first suggested when it was recognized that aspects of the structures of Bcl-2 and Bax are reminiscent of the pore-forming membrane translocation domains of colicins and diphtheria toxin (Muchmore et al., 1996; Suzuki et al., 2000). Indeed, Bcl-2 proteins do have ion channel-forming abilities (reviewed in (Desagher and Martinou, 2000)). Formation of channels sufficiently large to allow protein efflux may only be possible, however, upon formation of oligomeric structures in membranes, a property that is exhibited by Bax (Antonsson et al., 2000; Gross et al., 1998). Bax or Bak are capable of forming pore independently of any of the PT pore components but instead assisted by activated Bid (tBid) (Desagher et al., 1999; Eskes et al., 2000; Wei, 2000). tBid-induced

mitochondrial outer membrane permeabilization that is unaffected by PT pore inhibitors has been reported (Shimizu and Tsujimoto, 2000). Another study yielded slightly different results by revealing that Bax oligomers alone reconstituted into liposomes can produce cytochrome c-permeant pores (Saito et al., 2000). The existence of parallel Bid-dependent and –independent pathways for Bax insertion into the mitochondrial outer membrane is further supported by experiments conducted with Bid-null fibroblasts (Ruffolo et al., 2000).

At present it is uncertain which of these models most closely describes the physiological processes involved in mitochondria-assisted cell suicide. It may be that all of these models apply under distinct conditions. However, there can be no doubt that mitochondria play a key role in diverse cell death processes, including those accompanying virus infection.

## Viruses and apoptosis

The precise nature of the 'danger signals' that allow cells to sense infection and consequently, the need to initiate apoptosis, are varied (Everett and McFadden, 1999) but apoptotic cascades appear to be unavoidably activated by most viral infections.

The events surrounding the adsorption and entry of viral particles may constitute extrinsic apoptotic signals. Attachment of non-infectious, conformationally intact virions of bovine herpesvirus 1 (BHV-1), herpes simplex virus (HSV), human immunodeficiency virus (HIV), reovirus and vaccinia virus to the surface of cells has been shown to be pro-apoptotic (Esser et al., 2001; Hanon et al., 1998; Ramsey-Ewing and Moss, 1998; Tyler et al., 1995; Zhou et al., 2000). It is not known whether a receptor is involved in these examples of virus entry-mediated apoptosis.

Receptor-mediated death signaling does appear to be triggered during entry of Avian-Leukosis-Sarcoma virus, which attaches to cells by means of a TNFR-related protein, CAR1 (Brojatsch et al., 1996). Engagement of this receptor by the viral envelope protein is sufficient to initiate apoptosis, although the exact details of the mechanisms involved await further investigation. An interesting modification of death receptor stimulation occurs during hepatitis C virus infection. The core protein expressed by this virus contains a death domain and, in different experimental systems has been shown to bind to the death domain-containing cytoplasmic domains of either Fas (Hahn et al., 2000) or TNFR1 and FADD (Zhu et al., 1998; Zhu et al., 2001). Core protein binding is found to enhance Fas or TNF death signaling downstream of these respective death receptors under certain conditions. Therefore, although the virus does not actually initiate an extrinsic signal, it effectively induces constitutive activation of death receptor signals, thereby augmenting apoptosis of the infected cell. Sindbis virus entry into cells can also constitute an extrinsic signal (Jan et al., 2000). In this case, fusion of the virus particle to the plasma or early endosome membrane activates acidic sphingomyelinase also contained within these membranes. Activation of this enzyme results in the production of ceramide that, in turn, activates the ceramide-induced apoptotic pathway.

Upon entry into cells, viruses may stimulate a diversity of intrinsic cell death pathways. Viral replication strategies that trigger nuclear sensors, such as p53, designed to detect genotoxic damage or inappropriate proliferation signals, are well documented (reviewed in (Neil et al., 1997)). Numerous viruses trigger apoptosis by initially activating the double stranded RNA-activated protein kinase R (PKR) (reviewed in (Kaufman, 1999). Influenza A virus (Balachandran et al., 2000)) and Encephalomyocarditits virus (Yeung et al., 1999) are included in this

category. Increased production of viral proteins may also trigger the endoplasmic reticulum-dependent stress response and initiate apoptosis. For example, respiratory syncytial virus infection triggers this response which leads to the activation of caspase-12 and downstream effector caspases (Bitko and Barik, 2001).

The apoptotic cascades initiated by the extrinsic and intrinsic signals described above may converge on the mitochondrial cell death control point. In addition, this critical junction for cell death signaling cascades may also serve as cellular sensor that is triggered by viral proteins directly (Everett and McFadden, 2001). This concept is discussed in greater detail later in this chapter.

## Viral anti-apoptotic proteins

As a consequence of their inherent pro-apoptotic potential, many viruses have evolved highly efficient strategies to counteract cellular sensors designed to detect infection. Viral inhibitors of some of these sensors, such as p53 and PKR are well documented (Kaufman, 1999; Neil et al., 1997). In addition, viral strategies aimed at subverting key molecular components within apoptotic pathways namely, death receptors, caspases and Bcl-2 family members, have also been identified (reviewed in (Alcami and Koszinowski, 2000; Roulston et al., 1999; Tortorella et al., 2000)). Selected examples will be discussed in this section and are included in Table 1.1.

### Inhibition of death receptor signaling

Death receptor signaling is a prominent target for many viruses and two main strategies are employed to disarm this pathway. Specifically, viral

**Table 1.1 Viral anti-apoptotic proteins[a]**

| Mechanism | Protein | Virus | Reference |
|---|---|---|---|
| **Death Receptor Inhibitors** | | | |
| Receptor homologs | M-T2 | Myxoma virus | (Schreiber et al., 1997) |
| | Crm B, C and D | Cowpox virus | (Hu et al., 1994; Loparev et al., 1998; Smith et al., 1996) |
| Receptor downregulator | RID complex | Adenovirus | (Elsing and Burgert, 1998; Shisler et al., 1997; Tollefson et al., 1998) |
| v-FLIPs | E1.1 (BORFE2) | Bovine herpesvirus 4 | (Hu et al., 1997; Thome et al., 1997; Wang et al., 1997) |
| | E8 | Equine herpesvirus-2 | (Bertin et al., 1997; Hu et al., 1997; Thome et al., 1997) |
| | ORF71 | Herpesvirus saimiri | (Thome et al., 1997) |
| | ORF71/K13 | Human herpesvirus 8 | (Thome et al., 1997) |
| | MC159/MC160 | Molluscum contagiosum virus | (Bertin et al., 1997; Hu et al., 1997; Thome et al., 1997) |
| TNFR interaction | Core protein | Hepatitis C virus | (Ray et al., 1998) |
| FADD interaction | E1B19K | Adenovirus | (Perez and White, 1998) |
| TRADD/TRAF interaction | LMP-1 | Epstein-Barr virus | (Devergne et al., 1996; Kaye et al., 1996; Sandberg et al., 1997) |

**Table 1.1** Continued

| | | | |
|---|---|---|---|
| **Caspase inhibitors** | | | |
| Caspase inhibitor | p35 | Baculovirus | (Bump et al., 1995; Clem et al., 1991; Kamita et al., 1993; Xue and Horvitz, 1995) |
| Pro-caspase inhibitor | vCIA | Human cytomegalovirus | (Skaletskaya et al., 2001) |
| Serpin | CrmA | Cowpox virus | (Tewari et al., 1995; Zhou et al., 1997) |
| | SPI-2 | Rabbitpox virus | (Macen et al., 1996) |
| | B13R/B22R | Vaccinia virus | (Dobbelstein and Shenk, 1996; Kettle et al., 1997) |
| IAP | IAP | Baculovirus | (Birnbaum et al., 1994; Crook et al., 1993; Manji et al., 1997) |
| **Bcl-2 family modulators** | | | |
| Bcl-2 homologs | E1B19K | Adenovirus | (Boyd et al., 1994; Rao et al., 1992) |
| | KSBcl-2 | Human herpesvirus 8 | (Cheng et al., 1997; Sarid et al., 1997) |
| | HVSBcl-2 | Herpesvirus saimiri | (Derfuss et al., 1998; Nava et al., 1997) |
| | BHRF-1 and BALF-1 | Epstein-Barr virus | (Henderson et al., 1993; McCarthy et al., 1996; Tarodi et al., 1994; Marshall et al., 1999) |
| | M11 | Murine gammaherpesvirus | (Wang et al., 1999) |
| | A179L/p21 | African swine fever virus | (Alfonso et al., 1996; Brun et al., 1996; Revilla et al., 1997) |
| | BORFB2 | Bovine herpesvirus | (Bellows et al., 2000) |
| Bak degradation | E6 | Human papillomavirus | (Thomas and Banks, 1999) |
| **Anti-oxidant** | | | |
| | MC066L | Molluscum contagiosum virus | (Shisler et al., 1998) |

[a]Based on (Roulston et al., 1999; Tortorella et al., 2000)

proteins inhibit either receptor-ligand interactions or signaling downstream of the activated receptor.

Receptor-ligand interactions at the cell surface are prevented by the secretion of virus-encoded soluble receptor homologs from infected cells. The best characterized of these is the TNF receptor homolog, M-T2, encoded by the myxoma poxvirus (Schreiber et al., 1996; Schreiber et al., 1997). M-T2 is secreted early during myxoma virus infection and is able to bind and inhibit TNF. TNF is therefore prevented from binding its cognate receptor on the surface of infected cells and initiating cell death. Cowpox virus employs a similar 'decoy' strategy and produces three TNFR homologs, CrmB, CrmC and CrmD each with different activity profiles against a number of cytotoxic cytokines (Hu et al., 1994; Loparev et al., 1998; Smith et al., 1996). Adenovirus uses a different approach for preventing the availability of cell surface death receptors. This virus selectively induces the internalization and lysosomal degradation of the Fas receptor and other TNFR subtypes such as the TRAIL receptor. This process is mediated by the RID complex of proteins encoded by the E3 region of the genome (Benedict et al., 2001; Elsing and Burgert, 1998; Shisler et al., 1997; Tollefson et al., 1998).

A number of other viruses prevent signaling downstream of activated death receptors through the activity of viral FLICE (caspase-8) inhibitory proteins (v-FLIPs). These v-FLIPs all contain regions homologous to the death effector domain (DED) required for recruitment of procaspase-8 by FADD to activated Fas-, TNF- or other death receptor complexes. By binding to the death receptor complex, the v-FLIPs prevent caspase-8 activation and several cellular proteins have since been found to have a similar mode of action (Tschopp et al., 1998). A number of herpesviruses express v-FLIPs (Bertin et al., 1997; Hu et al., 1997; Thome et al., 1997; Wang et al., 1997). The v-FLIPs E1.1 (BORFE2) encoded by bovine

herpesvirus 4 and E8 encoded by equine herpesvirus-2 both primarily bind to pro-caspase-8 and inhibit caspase recruitment and activation in response to death receptor signaling. ORF71 and ORF71/K13 are DED-containing proteins encoded by herpesvirus saimiri and human herpesvirus 8 respectively and appear to act in the same manner as other herpesvirus v-FLIPs. The molluscum contagiosum poxvirus encodes two v-FLIPS, MC159 and MC160 (Bertin et al., 1997; Hu et al., 1997; Thome et al., 1997). MC159 is the main biologically significant vFLIP and blocks Fas signaling by forming a multimeric complex that contains Fas, FADD and pro-caspase-8. MC160 can also bind to FADD and procaspase-8 but is degraded if not co-expressed with MC159 (Shisler and Moss, 2001). Signaling downstream of death receptors is also modulated by other viral proteins that do not contain DED motifs. Included in this category are the hepatitis C virus core protein that can inhibit TNF-mediated apoptosis by binding to the TNFR (Ray et al., 1998) and E1B19K that binds to trimerized FADD and interferes with a number of death pathways (Perez and White, 1998). LMP-1 of Epstein-Barr virus mediates the assembly of a novel form of the TNFR signaling complex that contains the adaptor molecules TRADD and TRAF. This LMP-1 protein assembly gives rise to an altered form of TNF receptor signaling (Devergne et al., 1996; Kaye et al., 1996; Kieser et al., 1999; Sandberg et al., 1997).

**Viral caspase inhibitors**

Caspases are responsible for actually orchestrating many of the biochemical changes that accompany apoptosis and, not surprisingly, several viruses have evolved strategies to counteract the effects of these proteases. The p35 protein encoded by baculovirus is a broad specificity caspase inhibitor that is able to bind and inhibit multiple vertebrate and invertebrate caspases (Bump et al., 1995; Clem et al., 1991; Kamita et al., 1993; Xue and Horvitz, 1995). In the context of mammalian caspases, p35

has the broadest known inhibitory capacity and can inhibit capases-1, -3, -6, -7, -8 and -10. Most caspase inhibitors, including p35, disable their target enzymes by undergoing cleavage and then forming a stable inhibitory complex with the active enzyme (Bertin et al., 1996; Bump et al., 1995; Nicholson, 1999). Recently, another caspase inhibitor with no known homologs and a novel mode of action has been described. The anti-apoptotic protein vCIA of HCMV has been shown to prevent cleavage and activation of procaspase-8 by binding to the pro-domain of the inactive enzyme. vCIA is not able to bind to active caspase-8 and is not a vFLIP as it cannot bind to other DED-containing proteins such as FADD. This protein therefore acts at the apex of death receptor signaling (Skaletskaya et al., 2001).

Inhibition of caspase-mediated cell death is also achieved by a related class of poxviral proteins most notably CrmA of cowpox virus (Enari et al., 1995; Los et al., 1995; Tewari et al., 1995). Based on homology, CrmA is a member of the serine proteinase inhibitor (serpin) family, but this protein is able to inhibit caspases and is therefore a cross-class inhibitor (Komiyama et al., 1994). CrmA is a potent inhibitor of caspase-1 (or ICE), the caspase that regulates the maturation of the pro-inflammatory cytokine, IL-1β (Ray et al., 1992). In addition, CrmA is an effective inhibitor of an array to apoptotic cascades as a result of its ability to counteract the initiator caspase, caspase-8 (Srinivasula et al., 1996; Zhou et al., 1997) and the pro-apoptotic cytotoxic T cell serine protease, granzyme B (Quan et al., 1995). Other poxviral proteins, Spi-2 of rabbitpox virus (Macen et al., 1996) as well as B22R and B13R of vaccinia virus (Dobbelstein and Shenk, 1996; Kettle et al., 1997) share homology with CrmA but their role in apoptosis is less well defined.

Another important group of caspase regulators are the inhibitor of apoptosis proteins (IAPs). IAPs were first discovered in baculoviruses and

several cellular homologs have since been identified (Birnbaum et al., 1994; Crook et al., 1993; Deveraux and Reed, 1999; Manji et al., 1997). Cellular IAP proteins have been characterized as inhibitors of pro-caspase-9 activation within the Apaf-1/cytochrome c complex. These IAPs are the target of Smac/Diablo, hence binding of IAPs by Smac/Diablo relieves the IAP-mediated repression of caspase-9, allowing activation of this caspase (Du et al., 2000; Liu et al., 2000; Verhagen et al., 2000; Wu et al., 2000). Three baculoviral IAPs have also been identified that specifically bind to caspase-9 and inhibit its activity (Huang et al., 2000; Huang et al., 2001). Unlike the cellular counterparts, these proteins are unable to bind to caspases-3 and -7.

**Viral inhibitors of cellular pro-apoptotic Bcl-2 proteins**

A number of viruses encode Bcl-2 homologs that can be identified based on the presence of BH domains. These viral proteins are all anti-apoptotic. These include E1B19K encoded by adenovirus (Boyd et al., 1994; Rao et al., 1992) and A179L/p21 encoded by African swine fever virus (Alfonso et al., 1996; Brun et al., 1996; Revilla et al., 1997). Numerous herpesviruses also encode Bcl-2 homologs including KSBcl-2 encoded by human herpesvirus 8 (Cheng et al., 1997; Sarid et al., 1997), HVSBcl-2 encoded by herpesvirus saimiri (Derfuss et al., 1998; Nava et al., 1997), BHRF-1 and BALF-1 encoded by Epstein-Barr virus (Henderson et al., 1993; Marshall et al., 1999; McCarthy et al., 1996; Tarodi et al., 1994) M11 encoded by Murine gammaherpesvirus (Wang et al., 1999) and BORFB2 encoded by bovine herpesvirus 4 (Bellows et al., 2000).

The viral Bcl-2 homologs are thought to mimic cellular pro-survival Bcl-2 proteins. Indeed, most of the viral proteins are able to heterodimerize with cellular apoptosis-promoting Bcl-2 family members. Also, as described already, the anti-apoptotic cellular proteins Bcl-2 and Bcl-$X_L$ have latent

pro-apoptotic properties. Cleavage of either of these proteins by caspases liberates a strongly pro-apoptotic C-terminal domain in a process reminiscent of Bid activation (Cheng et al., 1997; Clem et al., 1998). Interestingly the viral Bcl-2 proteins all lack BH3 domains and seem to escape conversion to pro-apoptotic molecules. The viral Bcl-2 proteins tested either failed to be cleaved by caspases (KSBcl-2, HVSBcl-2, BHRF-1 and BORFB2) or cleavage did not generate a pro-apoptotic product (M11) (Bellows et al., 2000).

Human papillomavirus (HPV) counteracts Bak-mediated apoptosis by a novel mechanism. The HPV E6 protein, although not a Bcl-2 family member, binds to Bak and accelerates the proteolytic degradation of this pro-apoptotic Bcl-2 family member (Thomas and Banks, 1999). This mechanism is thought to protect virus-infected skin cells from the effects of UV-induced apoptotic signaling mediated by Bak (Jackson et al., 2000).

It is interesting to note that a number of viral Bcl-2 homologs are encoded by viruses that also contain vFLIPs or other mechanisms to counteract death receptor function. This may indicate that viruses require inhibitors of multiple pathways to allow escape from the many facets of the host's apoptotic response designed to remove infected cells. Also, as with cellular Bcl-2 proteins, there is evidence that the viral homologs do not only function by heterodimer formation and may directly modulate mitochondrial cell death processes. This is discussed later in this chapter.

**Oxidative stress prevention**

Molluscum contagiosum virus encodes a glutathione peroxidase, MC066L, that uses selenocysteine and antagonizes UV- and hydrogen-peroxide-induced apoptosis (Shisler et al., 1998). This mechanism for scavenging reactive oxygen metabolites perhaps provides the virus with a

selective advantage needed for replication in dermal layers exposed to sunlight.

Apart from the important molecular targets for viral modulators of apoptosis discussed in this section, a new emerging theme appears to be the ability of viruses to modulate the important convergence point of many death signals that is centered on the mitochondria (Boya et al., 2001; Everett and McFadden, 2001; Everett and McFadden, 2001). Viral proteins are extremely selective and efficient with respect to the cellular processes they target and are beginning to serve as valuable tools for exploring the complex process of mitochondria-mediated cell death.

## Modulation of the mitochondrial apoptotic checkpoint by viral proteins

A growing number of viral apoptotic modulators that influence mitochondrial cell death pathways can be identified (Table 1.2). In view of the ability of cellular Bcl-2 proteins to modulate the mitochondrial cell death processes, viral Bcl-2 homologues are the most likely candidates for modulating this checkpoint. Indeed, certain viral Bcl-2 homologs have a direct, proximal effect on mitochondrial function when expressed in cells independently of other viral proteins.

### Viral Bcl-2 homologs

The BHRF-1 protein of Epstein-Barr virus (EBV) is a functional anti-apoptotic Bcl-2 homolog that heterodimerizes with Bcl-2 and Bak (Nava et al., 1997) and was initially shown to protect cells from pro-apoptotic stimuli such as serum starvation, ionomycin treatment (Henderson et al., 1993), growth factor withdrawal (Takayama et al., 1994) anti-cancer agents and heterologous virus infection (Tarodi et al., 1994). A related protein,

**Table 1.2 Viral proteins that modulate the mitochondrial apoptotic checkpoint**

| Protein | Virus | Role in Apoptosis | Reference |
|---|---|---|---|
| **Bcl-2 homologs** | | | |
| BHRF-1 | Epstein-Barr virus | Inhibition | (Dumont et al., 1999) |
| | Herpesvirus papio | Inhibition | (Meseda et al., 2000) |
| E1B19K | Adenovirus | Inhibition | (Han et al., 1998; Perez and White, 2000) |
| **Bcl-2 homologs (presumed mitochondrial modulators)** | | | |
| BALF-1 | Epstein-Barr virus | Inhibition | (Marshall et al., 1999) |
| HVS-Bcl-2 | Herpesvirus saimiri | Inhibition | (Derfuss et al., 1998) |
| KSBcl-2 | Human herpesvirus 8 | Inhibition | (Cheng et al., 1997; Sarid et al., 1997) |
| **Mitochondrial modulators** | | | |
| p13$^{II}$ | Human T cell leukemia virus | Unknown | (Ciminale et al., 1999) |
| Apoptin | Chicken anemia virus | Induction | (Danen-van Oorschot et al., 2000) |
| E4orf4 | Adenovirus | Induction | (Livne et al., 2001) |
| Tat | Human immunodeficiency virus | Induction | (Kruman et al., 1998; Macho et al., 1999) |
| **PT pore modulators** | | | |
| HBx | Hepatitis B virus | Induction/inhibition, binds VDAC | (Diao et al., 2001; Rahmani et al., 2000) |
| vMIA | Cytomegalovirus | Inhibition, binds ANT | (Goldmacher et al., 1999) |
| Vpr | Human immunodeficiency virus | Induction/inhibition, binds ANT | (Jacotot et al., 2000) |
| M11L | Myxoma virus | Inhibition, binds PBR | (Everett et al., 2000) |

hpvBHRF-1, encoded by the related virus Herpesvirus papio, also has anti-apoptotic properties as demonstrated by its ability to protect cells from the genotoxic agent *cis*-platin (Meseda et al., 2000). Both BHRF-1 and hvpBHRF-1 proteins localize to mitochondria (Hickish et al., 1994) (Meseda et al., 2000), and are presumed to modulate the mitochondrial cell death control point. Other studies also support a model in which the protective effects of BHRF-1 are centered on mitochondria. For example, a truncated form of the protein lacking the C-terminal transmembrane domain has impaired survival-promoting activity (Theodorakis et al., 1996). Also, BHRF-1 is able to counteract apoptosis induced by reactive oxygen species, which act to disrupt mitochondrial integrity (Dumont et al., 1999). This same study also revealed that the E1B 19K protein encoded by adenovirus has the same ability to counteract the effects of reactive oxygen species.

The E1B19K protein of adenovirus is also a functional Bcl-2 homolog that was first shown to counteract the pro-apoptotic effects associated with expression of the E1A transforming protein of adenovirus or TNF treatment (Rao et al., 1992; White et al., 1992). E1B19K does not normally associate with mitochondria and was originally thought to exert its protective effects by binding and inhibiting pro-apoptotic BH3-containing Bcl-2 proteins such as Bax, Bak and NBK/Bik (White, 1998). However, it has subsequently been shown that, in response to the pro-apoptotic effects of Bax over-expression, E1B19K undergoes relocalization so as to interact with Bax in the mitochondrial outer membrane and provides a protective effect against Bax-induced mitochondrial $\Delta\Psi_m$ loss and apoptosis (Han et al., 1998). Interestingly, E1B19K also relocalizes and interacts with Bax following TNF-mediated caspase-8 activation, but only after Bax undergoes a conformational change induced by tBid. The E1B19K interaction with the pro-apoptotic form of Bax inhibits downstream

apoptotic events such as cytochrome c release and activation of caspases-9 and -3. (Perez and White, 2000).

Other viral anti-apoptotic Bcl-2 homologs are predicted to impact the mitochondrial cell death checkpoint, although they have not been formally characterized with respect to mitochondrial function. For example, Epstein-Barr virus encodes BALF-1, a second Bcl-2 homolog related to the BHRF-1 protein also encoded by this virus. BALF-1 is able to interact with Bax and Bak and prevents mitochondrial changes following induction of apoptosis by campothecin or Fas in combination with γIFN (Marshall et al., 1999). BALF-1 is presumed to modulate the mitochondrial control point based on its high degree of homology with BHRF-1.

The herpesvirus saimiri-encoded Bcl-2 homologue (HVS-Bcl-2) is another anti-apoptotic regulator and is capable of heterodimerization with Bcl-2, Bcl-$X_{L/S}$, Bax and Bak. This protein is thought to prevent cell autonomous apoptosis when expressed during the lytic cycle of infection and is known to prevent apoptosis induced by autologous virus infection (Nava et al., 1997). Stable expression of HVS-Bcl-2 alone is able to protect Jurkat T cells from a spectrum of apoptotic pathways triggered by Fas ligation, dexamethasone treatment, DNA damage or oxygen radical production (Derfuss et al., 1998). The protective effects of this viral Bcl-2 protein are apparently centered on mitochondria and include prevention of $\Delta\Psi_m$ loss, cytochrome c translocation into the cytosol and caspase-3 activation.

KSBcl-2 is a functional Bcl-2 homologue of human herpesvirus 8 that is able to prevent apoptosis mediated by autologous virus infection and Bax over-expression (Cheng et al., 1997; Sarid et al., 1997). This protein does not homodimerize or form heterodimers with other known Bcl-2 homologs but can be detected in punctate cytoplasmic membrane structures. This intracellular distribution suggests that KSBcl-2 localizes to

mitochondria and, by implication, may modulate the function of these organelles.

**Other Viral Proteins that modulate Mitochondria**

There is evidence that viral proteins that are not Bcl-2 homologs also influence the mitochondrial cell death control point. In many cases the ability of these proteins to directly impact mitochondria has not formally been shown but is strongly implied by available experimental evidence. Several examples of individual viral proteins that are implicated in promoting or inhibiting cell death mediated by mitochondria are discussed in this section.

The $p13^{II}$ protein of Human T cell leukemia/lymphotrophic virus type 1 (HTLV-1) contains a novel 10 amino acid targeting signal near the N-terminus that directs this protein to mitochondria (Ciminale et al., 1999; D'Agostino et al., 2000). $p13^{II}$ expression induces mitochondrial swelling and clustering as well as $\Delta\Psi_m$ loss. However, cytochrome c redistribution is not observed and the role of this protein in apoptosis modulation is yet to be established (Ciminale et al., 1999). The $p13^{II}$ protein that was used in these studies is the long isoform. A different, short isoform that is truncated at the C terminus, is expressed by virus strains that are unable to cause persistent infection (perhaps as a result of their inability to prevent apoptosis). Interestingly this short isoform, unlike the long isoform, has an increased capacity to bind to cellular proteins and specific interactions with homologs of adenylate kinase and an actin-binding protein homolog have been shown (Hou et al., 2000). It would be interesting to ascertain whether this isoform is able to induce mitochondrial changes and, together with its ability to bind to cellular proteins, has pro-apoptotic properties.

The apoptosis-inducing protein, Apoptin, of Chicken anemia virus induces cell death in a caspase-dependent manner. However, in the presence of caspase inhibitors, this protein is able to induce mitochondrial $\Delta\Psi_m$ loss and cytochrome c redistribution as a late event following apoptosis induction. These caspase-independent effects mediated by Apoptin may reflect activation of a default mitochondrial cell death pathway by this protein (Danen-van Oorschot et al., 2000).

Like Apoptin, the E4orf4 protein of Adenovirus is also thought to activate both caspase-dependent and -independent cell death pathways and is only functional in transformed cells. In one study of caspase-independent cell death, the pro-apoptotic effects of E4orf4 expression were associated with $\Delta\Psi_m$ loss and could be blocked by Bcl-2/Bcl-$X_L$ (Lavoie et al., 1998). In another study of caspase-dependent cell death, E4orf4 was found to trigger the Fas death receptor pathway by inducing caspase-8 activation and this induced cytochrome c redistribution. Interestingly, mitochondrial generation of reactive oxygen species was shown to be an essential component of death signaling by this protein, but caspase-9 activity downstream of mitochondria was not required, and caspase-3 was not activated in all cell types (Livne et al., 2001). These observations suggest that the post-mitochondrial death signaling induced in this system can be caspase-independent.

HIV encodes a number of apoptosis-modulatory proteins that trigger mitochondrial cell death processes (Badley et al., 2000; Basanez and Zimmerberg, 2001; Ferri et al., 2000). The best characterized of these are Vpr (discussed in the next section) and Tat. Tat functions as a transactivator for viral gene expression, but has also been implicated in triggering apoptosis by a variety of mechanisms including the mitochondrial cell death pathway. Specifically, exogenous addition of Tat to hippocampal neurons is reported to induce caspase activation, elevated

cytoplasmic and mitochondrial calcium levels, an accumulation of mitochondrial reactive oxygen species and $\Delta\Psi_m$ loss (Kruman et al., 1998). The mitochondrial PT pore is implicated in Tat-mediated cell death as apoptosis in this context is prevented by cyclosporin A, a PT pore inhibitor. In another study, Tat expression was found to sensitize two leukemic cell lines to apoptosis mediated by serum withdrawal (Macho et al., 1999). Cell death was shown to be associated with an $\Delta\Psi_m$ loss and increased production of reactive oxygen species that preceded caspase-3 activation. In one of the cell lines, induction of apoptosis was temporally linked to a redistribution of Tat from the cytoplasm and nucleus to mitochondria. Involvement of the PT pore in Tat-induced cell death was revealed when protoporphyrin IX, a ligand of the PBR subunit of the PT pore, was found to synergize with Tat in apoptosis induction.

The examples described above suggest the importance of the mitochondrial cell death checkpoint during virus infection and seem to implicate the PT pore as a target for modulation by viral proteins. However, much of this evidence is indirect and involves the use of chemical agonists or antagonists of the pore. More direct evidence to support the importance of PT pore modulation in infected cells has been provided by reports that several viral proteins associate directly with components of the pore. The best documented of these are the HBx protein of Hepatitis B virus, which binds to the VDAC, and the vMIA protein of HCMV and the Vpr protein of HIV and, which each bind to the ANT.

**Direct regulation of the PT pore by viral proteins**

The HBx protein, a transcriptional activator, is clearly implicated in the development of Hepatitis B virus-induced hepatocellular carcinoma, however its role in apoptosis remains controversial as both pro- and anti-

apoptotic functions have been attributed to this protein (reviewed in (Diao et al., 2001)). Involvement of HBx in the mitochondrial apoptotic control point was revealed during two studies that characterized the ability of HBx to localize to mitochondria and bind to VDAC (Rahmani et al., 2000; Takada et al., 1999). In both of these studies, HBx over-expression in HuH7 human hepatocellular carcinoma cells induced apoptotic changes including perinuclear clustering of mitochondria, $\Delta\Psi_m$ loss and DNA fragmentation.

The modulation of apoptosis by HBx is thought to arise from the ability of this protein to regulate cellular signal transduction pathways, therefore the effect of HBx expression on cell survival can be expected to be dependent on the context of expression. Indeed, in contrast to the experiments described above, another recent study (Diao et al., 2001) showed that expression of HBx in human liver cell lines and primary hepatocytes protected these cells from several apoptotic stimuli including Fas, TNF and serum withdrawal. HBx could not counteract the effects of a variety of chemical apoptotic stimuli. Following Fas treatment, expression of HBx in mouse fibroblasts prevented caspase-8 and -3 activation and cytochrome c translocation into the cytoplasm in a manner dependent on up-regulation of the growth-stimulatory SAPK/JNK pathway. Nevertheless, in a different system, HBx stimulation of the SAPK/JNK pathway is also reported to sensitize cells to TNFα-mediated killing (Su and Schneider, 1997). The opposing effects of HBx suggest that this protein acts to promote survival stimuli rather than to antagonize apoptosis directly. In view of this proposed function, it would be interesting to ascertain how HBx is targeted to mitochondria and whether the function of HBx is dependent on its mitochondrial localization.

Recently, a protein expressed by a newly identified splice variant of the UL37 gene of Human cytomegalovirus (HCMV), designated vMIA, has

been shown to protect cells from Fas-, TNF- and doxorubicin-mediated apoptosis (Goldmacher et al., 1999). vMIA localizes to mitochondria and physically associate with the ANT subunit of the PT pore. Apoptotic events upstream of mitochondria such as caspase-8 activation or Bid cleavage are not inhibited by vMIA, but this protein is able to preserve ATP generation following apoptosis induction, inhibit the release of apoptogenic factors such as cytochrome c from mitochondria and prevent caspase-9 activation. These properties support the theory that vMIA acts by inhibiting apoptosis at the mitochondrial control point, presumably by modulating the PT pore. This mitochondrial site of action was further reinforced by the finding that deletion of amino acids 2-23 from the N-terminal domain abolishes the targeting and function of this protein (Goldmacher et al., 1999). These findings are intriguing because vMIA has no database homologues and no sequence similarity to any of the Bcl-2 family members, and yet is highly conserved amongst different strains of HCMV (Hayajneh et al., 2001). Two functional domains are completely conserved amongst all isolates studied. The first domain located between amino acids 2-34 is required for the targeting and anti-apoptotic function of the protein whereas the second domain, located near the C terminus only contributes anti-apoptotic activity. The evolution of vMIA may anticipate the existence a cellular protein with an analogous function that is yet to be identified and emphasizes the importance of the ANT in apoptosis

Vpr has been implicated in diverse processes including HIV replication, cell cycle regulation and differentiation (reviewed in (Basanez and Zimmerberg, 2001; Ferri et al., 2000)). Studies of apoptosis in cells expressing Vpr have yielded disparate results and recently a dual role for Vpr in modulating apoptosis has been proposed (Conti et al., 2000). Specifically, during the course of productive HIV infection of human T cell lines, Vpr expression at early times of infection is able to prevent

apoptosis mediated by TNF in combination with cycloheximide. In contrast, prolonged or elevated levels of Vpr expression potentiates apoptosis of infected cells.

Interestingly, addition of Vpr or a C-terminal Vpr peptide (Vpr 52-96) to intact cells has been shown to induce apoptosis associated with $\Delta\Psi_m$ loss, production of reactive oxygen species and release of mitochondrial proteins including cytochrome c and AIF (Jacotot et al., 2000). Pro-apoptotic effects are also observed when Vpr is added to purified mitochondria, indicating that mitochondria are the primary site of Vpr-induced apoptotic changes. In addition, transiently expressed Vpr or exogenously added protein localizes to mitochondria in a sub-population of cells. Involvement of the PT pore was suggested in this study by the observation that Bcl-2 and the PT pore inhibitors, cyclosporin A and bongkrekic acid inhibited these Vpr-induced effects on isolated mitochondria and in intact cells. Also, when liposomes reconstituted with isolated PT pore complexes were exposed to Vpr, increased liposome permeability was observed and this effect was inhibited by cyclosporin A or bongkrekic acid. This is interesting in light of the previously characterized inherent channel-forming ability of Vpr (Piller et al., 1998).

A direct role for Vpr in modulating the PT pore was revealed when it was shown that, when added to isolated mitochondria, Vpr associates with a complex containing VDAC and ANT. (Jacotot et al., 2000). Studies undertaken using purified protein have shown that Vpr binds primarily to ANT and this interaction can be inhibited by bongkrekic acid. The binding interface between Vpr and ANT has been characterized. The interacting domain on Vpr is present in the C-terminal portion of the protein and requires a highly conserved amphipathic alpha helical region containing critical arginine residues (Jacotot et al., 2001; Jacotot et al., 2000). The ANT binding interface centers on the N-terminal second regulatory loop

orientated towards the intermembrane space in mitochondria (Jacotot et al., 2001).

**Significance of viral protein modulation of mitochondrial death signals**

An emerging theme for the mitochondrial apoptotic modulators of viral origin is that their activity seems to be directed towards mimicing the activities of survival-promoting Bcl-2 proteins or modulating components of the PT pore. Most notably, viral proteins directed specifically towards apoptogenic proteins released from mitochondria, such as cytochrome c or AIF, have yet to be discovered. This may reflect the relatively recent appreciation that cytochrome c and AIF are important apoptotic cofactors. Alternatively, viruses may deliberately avoid interfering with the activity of proteins that are also required for normal cellular metabolic functions. By impacting the PT pore and modulating apoptosis without directly impairing such essential processes as electron transport, viruses may thwart any cellular anti-viral strategy aimed at limiting the energy supply.

Recent advances in the elucidation of the molecular players in apoptotic cascades have revealed a pivotal role for mitochondria as a control point for relaying and amplifying many of the cell death signals. With this knowledge, it has become apparent that diverse viral apoptotic modulators have independently evolved mechanisms to exploit this critical junction where numerous cell death pathways intersect. Viral proteins that directly modulate mitochondrial function will continue to be valuable experimental tools for dissecting cell death pathways, particularly in terms of the 'cause' vs 'effect' controversy that surrounds the precise role of the PT pore complex during apoptosis.

## Myxoma virus and apoptosis modulation

A number of poxvirus-encoded proteins have been implicated in regulating apoptotic cascades, including several proteins expressed by myxoma virus, a Leporipoxvirus or poxvirus pathogen of rabbits (McFadden and Barry, 1998; Nash et al., 1999). Myxoma virus, is the causative agent of a lethal disease, myxomatosis, in the *Oryctolagus cuniculus* species of European or laboratory rabbit (DiGiacomo and Maré, 1994; Fenner and Myers, 1978). Myxomatosis is characterized by the formation of a primary lesion at the site of infection, followed by the development of disseminated internal and external lesions, suppression of cellular immunity and supervening Gram-negative bacterial infections of the respiratory tract. In a naïve European rabbit population, infection can result in greater than 99% mortality. Interestingly, myxoma virus only produces a benign, non-pathogenic disease within the phylogenetically distinct *Silvalagus* species of North and South American brush rabbit (*Silvalagus bachmani* and *Silvalagus braziliensis*). These rabbit populations serve as the reservoir for the virus and are relatively unaffected by infection, most likely as a result of co-evolution of host and virus over many generations and establishment of an equilibrium between the opposing influences of the need for the virus to replicate and the need for the immune system of this host species to limit virus infection.

An important determinant for successful myxoma virus replication is the ability of the virus circumvent the hosts' normally protective apoptotic response. Several myxoma virus apoptotic modulators that are expressed early during infection have been identified to date and include M-T2, M-T4, M-T5, and M11L. The important contribution made by these proteins towards virus virulence as well as their potential anti-apoptotic role was first revealed during the characterization of knockout virus strains that

had single targeted disruptions in the individual genes encoding these proteins. All of these knockout viruses produced attenuated disease phenotypes in rabbits and, during infection of RL-5 rabbit lymphocytes, were pro-apoptotic and were impaired in their ability to replicate (Barry et al., 1997; Macen et al., 1996; Mossman et al., 1996; Opgenorth et al., 1992; Upton et al., 1991). In addition, another myxoma virus protein, Serp2, was identified as a potential apoptotic modulator by virtue of its similarity to the Orthopoxvirus anti-apoptotic serpin, CrmA/SPI-2 (Petit et al., 1996). A Serp2 knockout virus was found to be competent for growth in RL-5 cells. However, in contrast to wild-type myxoma virus, this knockout virus produced attenuated disease symptoms and elevated apoptosis of lymphocytes in lymph nodes during infection of rabbits. These observations indicate that Serp2 plays an important role in the pathobiology of myxoma virus (Messud-Petit et al., 1998). It can be concluded that the expression of M-T2, M-T4, M-T5, Serp2 and M11L is required during infection of rabbit cells, particularly leukocytes, in order to prevent cell death and permit efficient virus replication.

The observation that expression of all of these proteins is required during infection implies that they each play a defined role and are non-redundant in function. M-T2 is a secreted TNF receptor homologue that binds that inhibits TNF under physiologically relevant conditions (Schreiber and McFadden, 1996; Schreiber and McFadden, 1994; Schreiber et al., 1996) and is therefore capable of abrogating apoptotic signals mediated by TNF. A separate, as yet undefined, anti-apoptotic role for the intracellular, non-glycosylated form of M-T2 has also been suggested (Schreiber et al., 1997; Sedger and McFadden, 1996). Serp-2 has been found to inhibit activation of the serine proteinase and pro-apoptotic effector produced by cytotoxic T cells, granzyme B. However, in comparison to CrmA/SPI-2, Serp2 has a considerably lower inhibitory activity and cannot prevent activation of pro-apoptotic caspases, suggesting that other intracellular targets for this

serpin are yet to be discovered (Turner et al., 1999). M-T4 is an endoplasmic reticulum-localized virulence factor (Barry et al., 1997; Hnatiuk et al., 1999) and M-T5 is an ankyrin repeat protein with homology to other poxvirus host-range genes and an ability to prevent a generalized inhibition of translation following infection (Mossman et al., 1996). The mechanisms whereby M-T4 and M-T5 inhibit apoptosis are currently unknown.

The M11L protein was first identified as an important determinant for the virulence of myxoma virus during the characterization of an M11L knockout virus (Opgenorth et al., 1992). Whereas infection of rabbits with myxoma virus produced the highly lethal symptoms of myxomatosis, infection with the M11L knockout virus merely elicited an attenuated, non-lethal disease phenotype. All the rabbits infected with the M11L knockout virus recovered fully and were resistant to subsequent challenge with wild-type myxoma virus. In addition, the lesions produced by the two viruses were markedly different (Table 1.3).

**Table 1.3 Comparison of vMyxlac and vMyxM11L⁻[a]**

| | Virus | |
|---|---|---|
| | **vMyxlac** | **vMyxM11L⁻** |
| Virulence in rabbits | > 99% mortality | no mortality |
| Lesion appearance | flat, hemorrhagic | raised edematous |
| Associated symptoms | 2° bacterial infection | inflammation |
| Apoptosis in RL-5 cells | low | high |

[a] Based on (Macen et al., 1996; Opgenorth et al., 1992)

Specifically, myxoma virus gave rise to hemorrhagic lesions prone to secondary bacterial infection whereas the M11L knockout virus produced raised edematous lesions that, upon histological analysis, displayed signs of increased inflammation and leukocyte infiltration. In contrast to myxoma virus, the M11L knockout virus also replicated poorly in primary rabbit splenocytes (Opgenorth et al., 1992) and, as discussed previously, infection of RL-5 rabbit lymphocytes with the two viruses revealed a potential anti-apoptotic role for M11L (Macen et al., 1996). Taken together, these findings suggested a model in which M11L could act as a virulence factor by preventing apoptosis of infected leukocytes.

M11L is a novel protein and currently there are no database homologues outside the poxvirus family (Figure 1.5). The protein is 166 amino acids in length, is expressed early in the virus replication cycle, is not glycosylated, and includes no distinct sequence motifs apart from a putative transmembrane domain at the carboxyl terminus (Graham et al., 1992). The preliminary analysis of M11L therefore provided little indication of the functional role of this protein or how it might impact apoptotic cascades, despite its profound contribution towards virus virulence. The objective of this research study was thus to characterize the properties and biological functions of M11L.

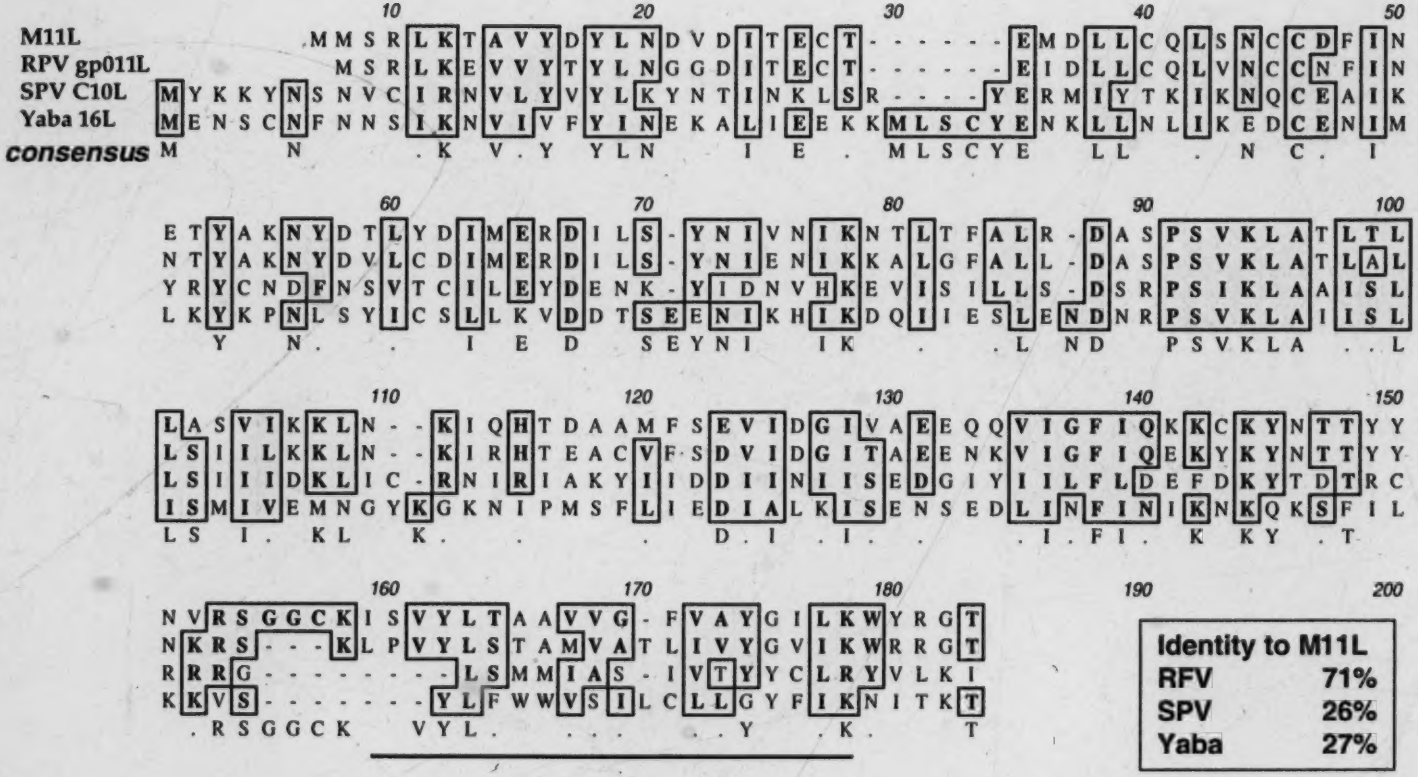


**Figure 1.5 M11L homologs.** Alignment between M11L and its homologs from rabbit fibroma virus (RFV), swinepoxvirus (SPV) and Yaba poxvirus (Yaba). The putative transmembrane region is underlined.

## Thesis Objectives

The studies described in this thesis were directed towards addressing three fundamental questions.

1. Is M11L a genuine anti-apoptotic protein with the ability to inhibit the transmission of apoptotic signals, even when expressed autonomously from other viral proteins?
2. What is the sub-cellular localization of M11L, what determines this localization and what are the functional consequences?
3. Does M11L interact with other cellular proteins and do these interactions determine the mechanistic aspects of M11L action?

# Chapter 2[2]

## M11L is anti-apoptotic

[2] The data included in this chapter have been published (Everett et al., 2000) or are submitted in: The Myxoma poxvirus protein, M11L, prevents apoptosis by direct interaction with the mitochondrial permeability transition pore. Helen Everett, Michele Barry, Siow Fong Lee, Christine Frantz, Luc G. Berthiaume, Grant McFadden and R. Chris Bleackley

## Introduction

Myxoma virus encodes several virulence factors that are proposed to have anti-apoptotic properties, as outlined in the previous chapter. One of these virulence factors is M11L, a protein whose potential role in apoptosis was revealed during the characterization of a highly attenuated myxoma virus mutant with a targeted disruption in the *M11L* gene (Opgenorth et al., 1992). Specifically, this M11L knockout virus was found to induce apoptosis when infecting RL-5 rabbit lymphocytes, whereas infection with an M11L-producing, control myxoma virus did not have this pro-apoptotic effect (Macen et al., 1996). These findings provided the first indication that the virulence properties of M11L could be related to the ability of this protein to prevent apoptosis and suggested a model in which, during the normal process of myxoma virus infection, M11L expression is required to prevent leukocyte cell death.

M11L is a novel protein with no known database homologs outside the poxvirus family (Figure 1.5) and no similarity to any described anti-apoptotic proteins. Therefore, the sequence of M11L gave no indication of the precise function of this protein despite its striking contribution towards virus virulence and possible anti-apoptotic role. The available data did not permit distinction between the proposal that M11L functions to prevent apoptosis directly or the alternate proposal that the anti-apoptotic properties of M11L are secondary to some other role during virus replication. To address the fundamental issue of whether M11L is anti-apoptotic, we investigated the ability of M11L to prevent cell death both in infected cells and when expressed autonomously from other viral proteins. M11L was, indeed, found to have anti-apoptotic properties when expressed in both contexts. In order to obtain definitive evidence that M11L is an apoptotic inhibitor, it was then necessary to demonstrate that this protein can directly block the molecular events associated with an

apoptotic cascade. It was therefore shown that M11L has the ability to block apoptotic events downstream of Bid cleavage and upstream of cytochrome c release. The apoptosis-inhibitory effects of M11L are therefore centered on the mitochondrial cell death control point.

## Results

### Myxoma virus infection protects cells from apoptotic stimuli other than infection

Previous experiments had revealed that expression of certain myxoma virus proteins, including M11L, is required to prevent apoptosis during infection (Macen et al., 1996). In order to confirm the anti-apoptotic effects of M11L, the level of DNA fragmentation that occurred during infection in the presence or absence of M11L expression was assessed. RL-5 rabbit T lymphocytes were mock-infected or infected with M11L-producing wild-type myxoma virus (strain Lausanne) or vMyxlac, or with the M11L knockout virus vMyxM11L⁻. As shown in Figure 2.1 (Panel A), cellular DNA fragmentation did not occur in mock-infected cells or in cells infected for 12 h with the M11L-producing viruses (Lanes 2-4). In contrast, DNA fragmentation, a hallmark of apoptosis that is discernable as a laddering pattern, occurred in RL-5 cells as a result of infection with the M11L knockout virus (Lane 5). This experiment therefore verified that M11L expression is required during infection of RL-5 lymphocytes in order to prevent virus-induced apoptosis.

These experiments did not address the question of whether the protective properties of M11L would also be effective against an exogenously added apoptotic agent, such as staurosporine. Staurosporine is a nonselective inhibitor of a diversity of kinases (Meggio et al., 1995; Ruegg and Burgess, 1989) and is an efficient general agent for the induction of apoptosis.

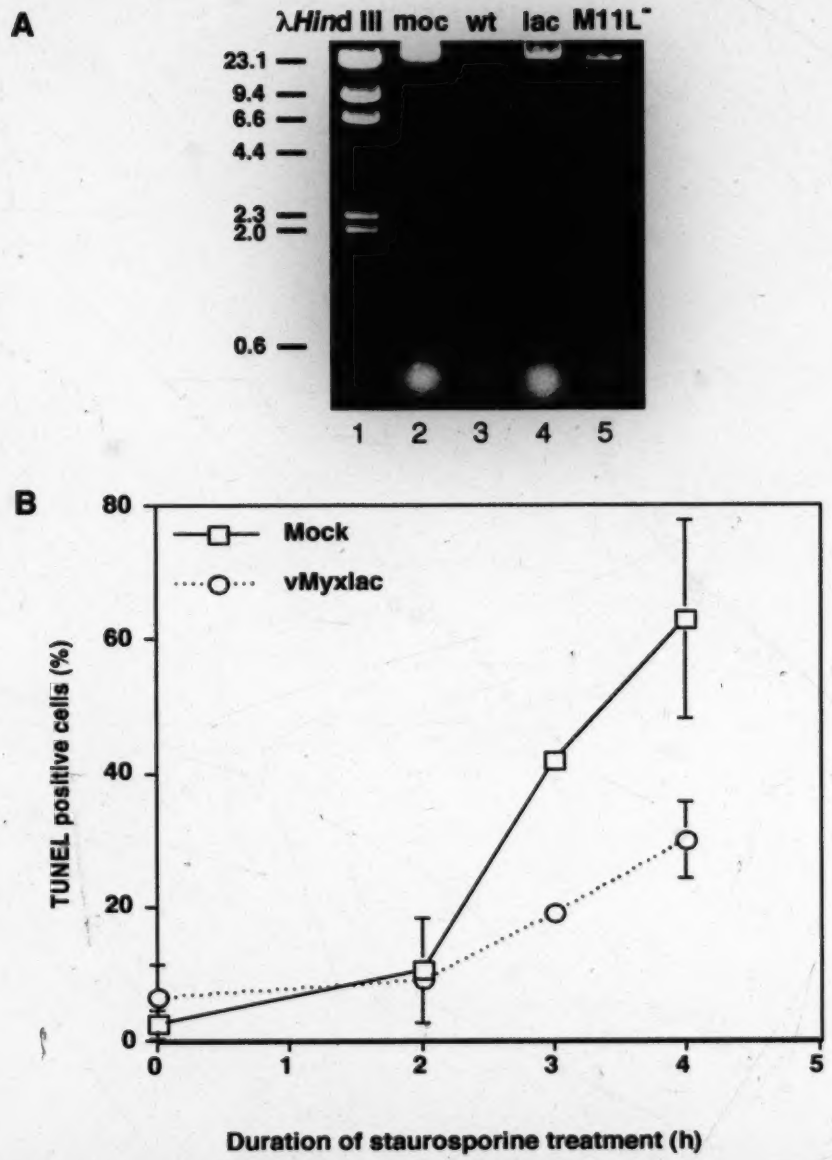


**Figure 2.1 Myxoma virus infection and M11L expression protects cells from apoptosis.** (A) Infection of RL-5 cells with wild-type myxoma virus (wt) or vMyxlac (lac), like mock infection (moc), did not induce DNA laddering. In contrast, M11L knockout virus (M11L⁻) infection did induce ladder formation and was therefore pro-apoptotic. (B) vMyxlac or mock-infected RL-5 cells were treated with staurosporine. The percentage of TUNEL positive (apoptotic) cells was elevated in mock infected cells relative to virus-infected cells.

However, despite the widespread use of staurosporine as an apoptosis-inducing agent, (e.g. (Bertrand et al., 1994; Jacobson et al., 1994) its precise mode of action is unknown. RL-5 cells, were mock-infected or infected with myxoma virus (vMyxlac) and 12 h post-infection apoptosis was induced by treatment with 2 μM staurosporine for up to 4 h. Apoptotic cells were identified using the TUNEL reaction to measure the characteristic elevation in the levels of nicked DNA. Following staurosporine treatment, the percentage of TUNEL positive (apoptotic) cells was found to be substantially higher in the mock-infected cell population than was the case with infected cells (Figure 2.1 Panel B). Thus, myxoma virus infection counteracts the pro-apoptotic effects of staurosporine.

**The M11L protein of myxoma virus is anti-apoptotic**

The findings described above raised the possibility that even when expressed autonomously from other viral proteins, M11L would be able to protect cells from apoptotic agents such as staurosporine. A Rat2 fibroblast cell line that constitutively expresses M11L (Rat2M11L) was therefore established using a retrovirus-based approach. A control cell line, Rat2puro, was established in parallel using the empty vector alone. M11L was detected by immunoprecipitation, SDS-PAGE and immunoblot analysis (Figure 2.2, Panel A). M11L was correctly expressed in control Rat2 cells infected with the M11L over-expressing virus, VVM11L (Lane 1) and in RatM11L cells designed to ectopically express the protein (Lane 2). No differences between M11L produced by ectopic expression or as a result of infection could be detected. In addition, M11L remained present in Rat2M11L cells for at least 5 h following treatment with staurosporine (Lane 4). There was no evidence that M11L underwent cleavage or degradation under these conditions. As expected, M11L was not detected in control Rat2puro cells (Lanes 6 and 8) or when control pre-immune

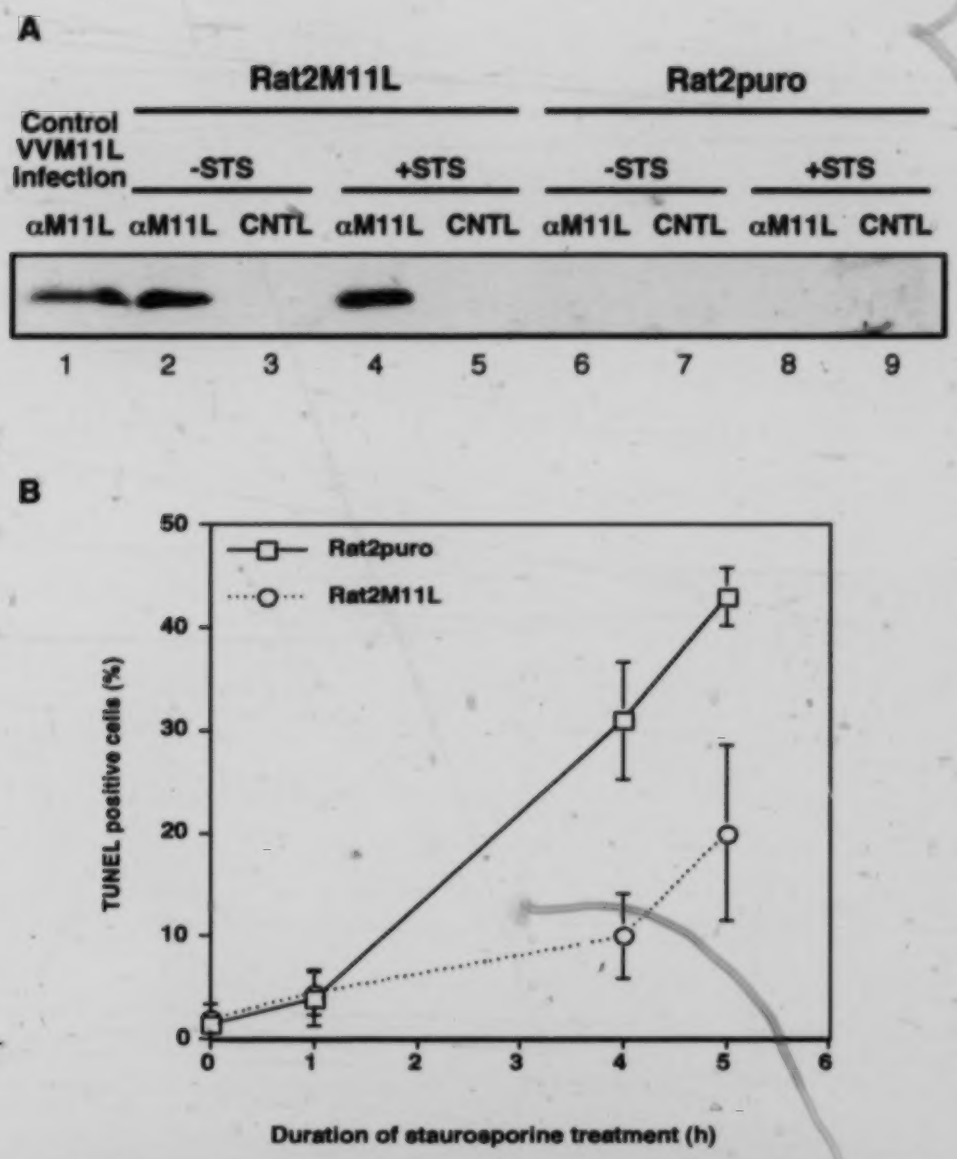


**Figure 2.2 Ectopic expression of M11L protects cells from staurosporine-mediated apoptosis.** (A) M11L was detected by immunprecipitation with an anti-M11L antibody (αM11L) or a control antibody (CNTL). Immunoblot analysis revealed that M11L is present as a stably expressed protein in Rat2M11L cells but not in control Rat2puro cells irrespective of staurosporine treatment (STS). (B) Rat2M11L cells displayed reduced TUNEL staining than Rat2puro cells following staurosporine-induced apoptosis.

rabbit serum was used to immunoprecipitate samples (Lanes 3, 5, 7 and 9). Therefore, it can be concluded that M11L is present and can be correctly detected when expressed in Rat2M11L cells.

The ability of staurosporine to induce apoptosis in both Rat2puro cells and Rat2M11L cells was monitored by measuring DNA fragmentation using the TUNEL assay. Whereas 2 μM staurosporine treatment for up to 5 h produced a steady increase in the levels of TUNEL positive Rat2puro cells, the same treatment of Rat2M11L cells produced a lesser increase in the percentage of TUNEL positive cells (Figure 2.2, Panel B). It could therefore be concluded that stable expression of M11L is able to protect cells from staurosporine-induced apoptosis. This result provided the first evidence that M11L is anti-apoptotic when expressed independently of other myxoma virus proteins and is able to function in cells derived from a species other than rabbit.

To confirm that M11L directly impacts an apoptotic cascade, it was necessary to determine whether M11L interferes with an end-stage apoptotic signal, such as the cleavage and activation of the effector caspase, caspase-3. To address this question, caspase-3 processing in Rat2puro and Rat2M11L cells was monitored by immunoblot analysis using an antibody directed against the large subunit of the active caspase. Treatment of Rat2puro cells with 5 μM staurosporine over a duration of 4 hours resulted in activation of caspase-3 as revealed by reduction in the amount of the 32 kDa pro-enzyme and appearance of the p19 cleavage product, a component of the active enzyme (Figure 2.3, Upper panel). In contrast, caspase-3 activation in Rat2M11L cells following the same treatment was substantially reduced (Figure 2.3, Lower panel), with only a low level of activation being apparent after 4 h treatment (compare Lane 4, Upper and Lower panel). These results therefore show that M11L impedes apoptotic signaling events upstream of caspase-3 activation.

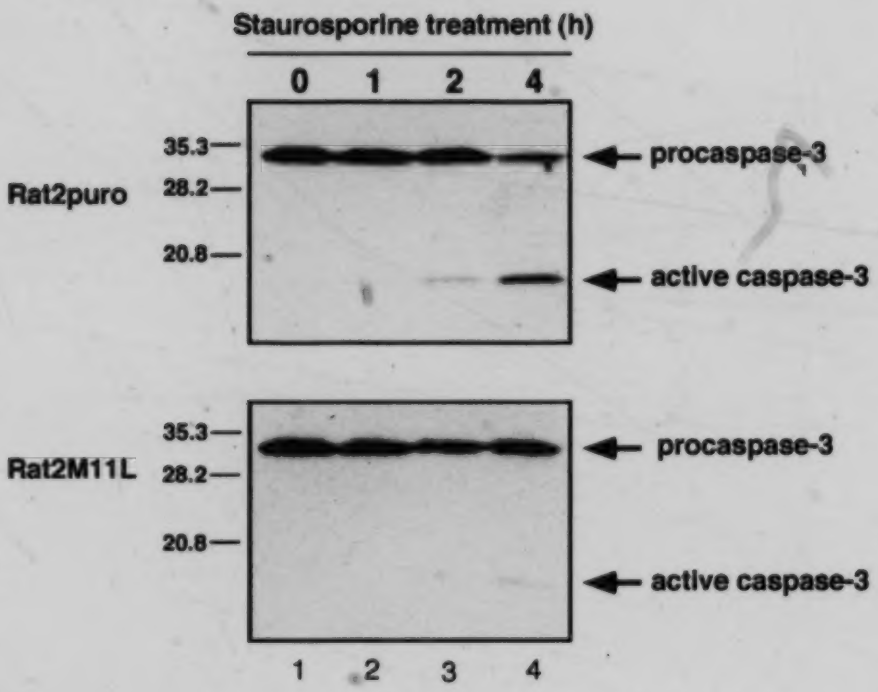


**Figure 2.3 M11L prevents procaspase-3 activation.** Control Rat2puro (top panel) and M11L-expressing Rat2M11L (bottom panel) cells were treated with staurosporine and caspase-3 was detected in whole cell lysates by SDS-PAGE and immunoblot analysis using an antibody directed against the large subunit of the active caspase. Cleavage of the 32-kDa procaspase-3 to produce the detectable 19-kDa component of the active caspase was observed in Rat2puro but not Rat2M11L cells.

**M11L inhibits apoptosis upstream of cytochrome c release and downstream of Bid cleavage.**

With the knowledge that M11L is anti-apoptotic and inhibits apoptotic signal transduction at some junction prior to caspase-3 activation, it was of interest to identify the precise step inhibited by M11L. A key event upstream of caspase-3 activation in many apoptotic pathways is the translocation of the apoptotic cofactor, cytochrome c, from the mitochondrial intermembrane space into the cytoplasm (reviewed in (Martinou and Green, 2001; Zamzami and Kroemer, 2001)). The effect of M11L on the relocalization of cytochrome c during apoptosis was therefore investigated. Rat2puro or Rat2M11L cells were treated with staurosporine for 2, 3 or 4 h or with DMSO solvent as a control, and digitonin lysates were prepared. Supernatant fractions containing cytosol were isolated and subjected to SDS-PAGE and immunoblot analysis using an antibody specific for cytochrome c. As shown in Figure 2.3 (Panel A), whereas DMSO treatment alone did not induce cytochrome c release from mitochondria in either cell line (Lanes 1 and 5) staurosporine treatment of Rat2puro cells triggered a rapid release of cytochrome c into the supernatant fraction that was detectable even 2 h post-treatment (Lanes 2-4). In contrast, following staurosporine treatment of Rat2M11L cells, the release of cytochrome c was barely detectable, even after 4 h (Lanes 6-8). This result indicates that M11L prevents cytochrome c translocation into the cytoplasm in response to staurosporine-induced apoptosis and led to the conclusion that M11L prevents apoptotic signals upstream of cytochrome c release.

Staurosporine-induced cytochrome c release from mitochondria is known to be preceded by activation of the pro-apoptotic Bcl-2 family member, Bid, in some systems (Desagher et al., 1999; Stepczynska et al., 2001). Bid becomes activated by proteolytic cleavage and liberation of a 15 kDa C-

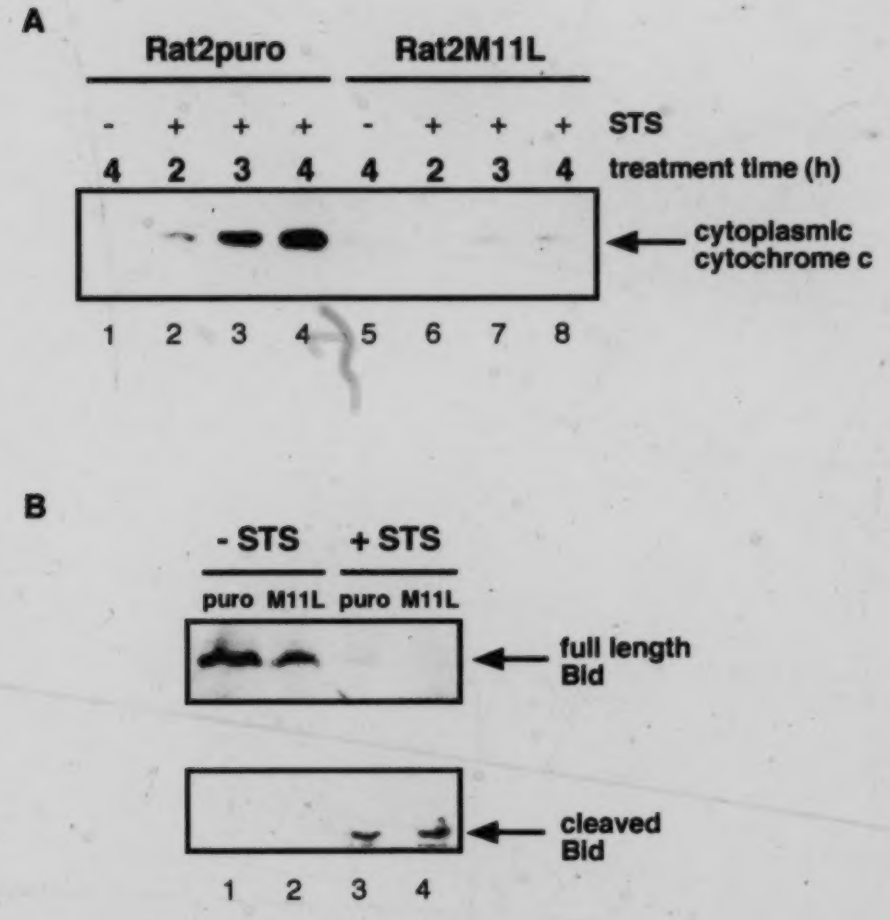


**Figure 2.4 M11L prevents apoptotic signaling upstream of cytochrome c release and downstream of Bid cleavage.** Rat2puro or Rat2M11L cells were treated with staurosporine (+STS) or DMSO solvent alone (-STS) as control. SDS-PAGE and immunoblot analysis allowed detection of (A) redistribution of cytochrome c to the cytosol in Rat2puro but not Rat2M11L cells. In both cell lines (B) cytosolic full length Bid (upper panel) could be detected in the absence of staurosporine treatment (-STS). Staurosporine-induced apoptosis (+STS) resulted in the generation of a Bid cleavage product that was identified in the pellet fraction (lower panel).

terminal effector fragment (tBid) that inserts into the mitochondrial outer membrane (Crompton, 2000; Harris and Thompson, 2000). To investigate the effects of M11L expression on Bid cleavage, Rat2puro and Rat2M11L cells were treated with 5 μM staurosporine or DMSO solvent for 3 h. The cells were then lysed in a digitonin lysis buffer, and pellet and supernatant fractions were isolated for SDS-PAGE and immunoblot analysis with an antibody directed against Bid. As shown in Figure 2.4 (Panel B), Bid remained uncleaved in the supernatant fractions of both cell lines treated with DMSO alone (Upper and Lower panels, Lanes 1 and 2). When both Rat2puro and Rat2M11L cells were treated with staurosporine, loss of full-length Bid and generation of a 15 kDa tBid cleavage product was observed (Upper and Lower panels, Lanes 3 and 4). Therefore M11L expression does not prevent the cleavage of Bid upstream of the mitochondrial apoptotic control point. This result, together with the finding that M11L does prevent cytochrome c translocation from mitochondria, supports the conclusion that M11L activity is centered on the mitochondrial apoptotic control point.

**M11L prevents mitochondria from undergoing a permeability transition following apoptosis induction**

Considering that M11L appeared to impede apoptotic signal transduction via mitochondria, the possibility that M11L could prevent direct mitochondrial effects following apoptosis induction, such as the loss of electrical potential difference across the inner membrane, merited investigation. The potentiometric dye, $DiOC_6$, provides an accurate measure of mitochondrial inner membrane potential ($\Delta\Psi_m$) because the fluorescence intensity of this dye following incorporation into mitochondria is proportional to $\Delta\Psi_m$ (Metivier et al., 1998). The mitochondrial membrane potential in Rat2puro and Rat2M11L cells was

measured as a function of $DiOC_6$ fluorescence in the presence or absence of staurosporine treatment.

Representative results from one of three experiments shown in Figure 2.5, reveal that the mitochondria in control cells from both cell lines stained brightly (Figure 2.5, Panels a and c). Loss of fluorescence was induced by the protonophore CCCP, a protonophore which induces collapse of the mitochondrial inner membrane potential (Figure 2.5, Panel a, insert), thereby verifying that $DiOC_6$ fluorescence provided an accurate measure of $\Delta\Psi m$ in these cells. When subjected to the pro-apoptotic effects of staurosporine, mitochondria in Rat2puro cells displayed a greater loss of that $DiOC_6$ fluorescence than Rat2M11L cells (Figure 2.5, Panels b and d). These results indicate that M11L expression can protect mitochondria from undergoing membrane potential loss following staurosporine-induced apoptosis.

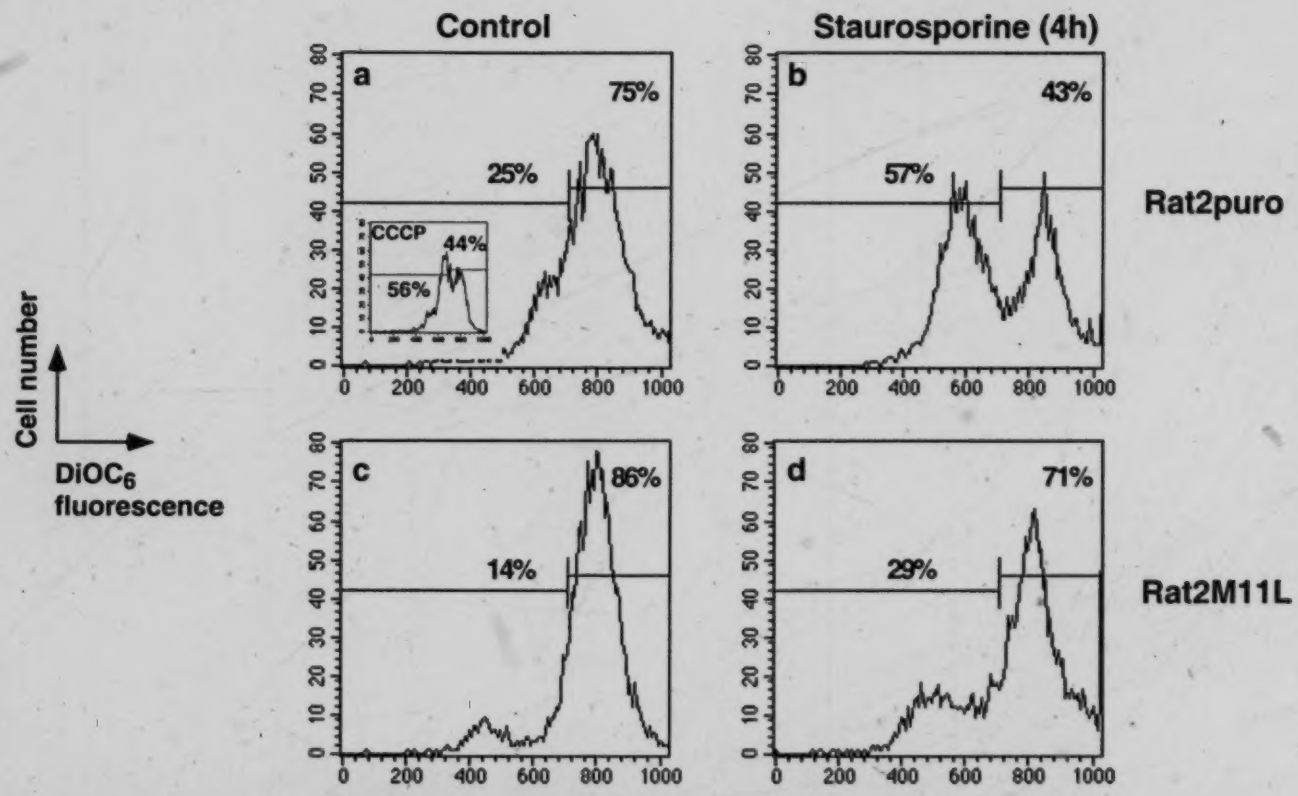


**Figure 2.5 M11L prevents mitochondrial ΔΨm loss.** Rat2puro (a, b) or Rat2M11L (c, d) cells were subjected to control (a, c) or staurosporine (b, d) treatment before staining with the potentiometric dye $DiOC_6$. Cells from both lines subject to control treatment (a, c) displayed intense staining indicating normal mitochondrial function. The protonophore CCCP markedly attenuated the fluorescent signal (a, insert). Staurosporine treatment resulted in attenuation of the signal produced by Rat2puro cells (b) but not Rat2M11L cells (d).

## Discussion

The unique role of M11L in the virulence of myxoma virus was indicated by two previous experimental observations. Firstly, in contrast to control virus, an M11L knockout myxoma virus elicited a markedly attenuated disease phenotype associated with unusual tumor-like lesions containing large numbers of infiltrating inflammatory cells (Opgenorth et al., 1992). Secondly, M11L was identified as a factor required to prevent apoptosis during myxoma virus infection of RL-5 lymphocytes *in vitro* (Macen et al., 1996). These observations suggested a model in which M11L acts as a virulence factor by virtue of its ability to prevent infected leukocytes from initiating a protective apoptotic response, thereby promoting viral replication.

The results described in this chapter indicate that M11L not only exerts a host cell-protective effect when the process of infection serves as the apoptotic trigger but also show that infection of cells with an M11L-producing virus prevents induction of apoptosis by an exogenously added agent, staurosporine. The effects of M11L are even more far ranging because, when this protein is expressed independently from other viral proteins, cells are also protected from the pro-apoptotic effects of staurosporine. In addition, M11L can prevent caspase-3 activation suggesting that this protein has a direct effect on a strategic step in an apoptotic cascade upstream of caspase-3. Hence M11L has the properties of a genuine anti-apoptotic protein.

In order to determine whether M11L can directly block the molecular events of an apoptotic cascade, the effect of M11L on the staurosporine-induced activation of apoptotic effectors upstream of caspase-3 was investigated. M11L was found to block cell death downstream of Bid cleavage and upstream of cytochrome c release demonstrating an ability

to inhibit propagation of apoptotic signals via the mitochondrial control point. Indeed, M11L had a direct effect on sparing mitochondrial function by preventing $\Delta\Psi m$ loss following staurosporine-induced apoptosis. In this respect, the properties of M11L are comparable to those exhibited by anti-apoptotic Bcl-2 proteins of cellular and viral origin as discussed in Chapter 1. In conclusion, the experimental results described in this chapter support an anti-apoptotic role for M11L that is centered on the mitochondrial apoptotic checkpoint.

## Materials and Methods

### Cells

RL-5 rabbit $CD4^+$ lymphocytes (NIH: AIDS research and reference reagent program) were maintained in RPMI medium (Gibco Life Technologies) supplemented with 10% fetal bovine serum (FBS, Gibco Life Technologies), 200 U/ml penicillin and 200 µg/ml streptomycin. Rat2puro and Rat2M11L rat fibroblasts were maintained in DMEM (Gibco Life Technologies) supplemented with 10% FBS, 200 U/ml penicillin 200 µg/ml streptomycin and 2.5 µg/ml puromycin.

### Virus infections

Virus infections were conducted using wild-type myxoma virus (strain Lausanne) or vMyxlac, a control recombinant myxoma virus (strain Lausanne) that has been described previously (Opgenorth et al., 1992). vMyxlac expresses the *Escherichia coli lacZ* gene from an intergenic region under the control of the vaccinia virus synthetic late promoter. RL-5 lymphocytes ($1x10^6$) were infected with vMyxlac at a multiplicity of infection (MOI) of 10 by incubating cells and virus at 37°C for 1 h in a total volume of 0.2 ml medium with constant agitation. The cells were then

added to 2 ml culture medium and incubated for a further 12 h before apoptosis induction.

**Induction and measurement of apoptosis**

Apoptosis was induced by infection (as described above) or by addition of staurosporine (Sigma) to the cell culture medium at a final concentration of 2 μM.

DNA fragmentation was assessed 12 h post-infection by lysing the cells in SDS lysis buffer (1% SDS, 100mM NaCl, 1 mM EDTA, 10 mM Tris pH 7.5). The lysates were treated with 0.5 mg/ml proteinase K (Sigma) for 1 h at 55°C and then with 0.5 mg/ml RNase A for 1 h at 37°C. The DNA was precipitated with 2.5 volumes of 95 % ethanol, resuspended in 10 mM Tris pH 8.0 and subjected to 1% agarose gel electrophoresis in the presence of 0.5 μg/ml ethidium bromide.

Apoptosis was induced by addition of staurosporine (Sigma) to the cell culture medium at a final concentration of 2 μM. Apoptotic cells were identified by monitoring their content of elevated levels of nicked DNA produced as a result of the characteristic genomic fragmentation which occurs during apoptosis. DNA fragmentation was measured using the TUNEL method described previously (Sgonc et al., 1994). Briefly, $1x10^6$ cells were harvested, washed in PBS and fixed in 2% paraformaldehyde /PBS at room temperature for 30 min. Cells were then washed in PBS containing 1% fetal bovine serum and permeabilized in 100 μl cold permeabilization buffer (0.1% Triton X-100 and 0.1% sodium citrate) for 2 min prior to washing. The cells were then incubated at 37°C for 1 h in 30 μl of reaction mix containing 25 mM Tris pH 6.6, 200 mM potassium cacodylate, 0.25 μg/μl BSA, 12 μM Fluorescein-dUTP, 0.6 μM dATP, 5 mM $CoCl_2$ and 0.5 U/μl of terminal deoxynucleotidyl transferase (Boehringer

Mannheim). After labeling, the cells were washed and analyzed by flow cytometry using a Becton-Dickinson FACScan flow cytometer equipped with an argon-ion laser with 15mW of excitation at 488nm. The fluorescent signal was acquired at logarithmic gain on 10 000 cells per sample through the FL1 channel equipped with a 530nm filter (30nm band pass) and data for the gated cell population were used.

**Generation of Rat2puro and Rat2M11L cells**

The *M11L* coding sequence was amplified by PCR using the primers 5'Eco (GCTAGAATTCATGATGTCTCGTTTAAAGAC) and 3'Sal (CGTAGTCGACTAGGTCCCTCGGTACC) and cloned into the T-tailed vector pT7blue (Novagen) to generate the pT7M11L plasmid. The *M11L* gene was sub-cloned as a *Bam*HI and *Sma*I fragment into the multiple cloning site of the murine leukemia virus-based vector pBabePuro (Morgenstern and Land, 1990) to produce the vector pBabePuroM11L. The plasmids pBabePuro and pBabePuroM11L were transfected into BOSC 23 cells and packaged into ecotropic virus particles as described (Pear et al., 1993). After infection of Rat2 fibroblasts with the ecotropic virus, pooled clones of cells that had stably incorporated provirus were selected on the basis of ability to grow in DMEM growth medium containing 2.5 μg/ml puromycin. The cell lines were designated Rat2M11L or Rat2puro depending on the vector used for their generation.

**Immunoprecipitation and detection of M11L**

Immunoprecipitation of M11L was achieved by resuspension of harvested cells in 1 ml in NP40 lysis buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 2 mM EDTA and 1 mM PMSF) and agitation at 4°C for 30 min. Insoluble material was pelleted at 4°C by centrifugation at 15000g for 10 min. The supernatant was removed and

10µl of rabbit polyclonal anti-M11L antibody (Graham et al., 1992) was added. The samples were incubated at 4°C with constant agitation for 2 h and 20 µl of a 50% slurry of Protein A-Sepharose beads (Pharmacia) was added before further incubation for 1 h. The beads were then pelleted by low speed centrifugation, washed in lysis buffer, and boiled in SDS sample buffer for 5 min. M11L was detected using the primary rabbit polyclonal anti-M11L antibody at a dilution of 1:500 and a secondary ProteinA-HRP conjugate (Pierce) at a dilution of 1:10000 followed by ECL detection (Amersham).

**Measurement of caspase-3 activation**

Rat2puro and Rat2M11L cells ($1x10^6$) were incubated with 5 µM staurosporine (Sigma) for up to 4 h to induce apoptosis. Cleavage and activation of caspase-3 was detected by SDS-PAGE and immunoblot analysis as described (Atkinson et al., 1998) using an antibody directed against the large subunit of the active enzyme (kindly provided by Dr. D. Nicholson).

**Detection of cytochrome c translocation and Bid cleavage**

Cytochrome c translocation into the cytoplasm of Rat2puro or Rat2M11L cells was analyzed by treatment of cells ($1x10^6$) with 5 µM staurosporine for 2, 3 or 4 h, or with an equivalent amount of DMSO for 4 h as a solvent control. In order to investigate Bid cleavage, Rat2puro or Rat2M11L cells ($5x10^6$) were treated with 5 µM staurosporine for 3 h, or treated with an equivalent amount of DMSO for 3 h as a solvent control. Cells were harvested by trypsinization and lysed at 4°C in 100 µl digitonin lysis buffer (75 mM NaCl, 1 mM $NaH_2PO_4$, 8 mM $Na_2HPO_4$ 250 mM sucrose and 95 µg/ml digitonin). Pellet and supernatant fractions were isolated by centrifugation of samples at 15000g and 4°C for 15 min and subjected to

SDS-PAGE and immunoblot analysis. Cytochrome c present in the supernatant fractions was detected using an anti-cytochrome c antibody (Pharmingen) and Bid present in supernatant and pellet fractions was detected using a rabbit polyclonal anti-Bid antibody (Wasilenko et al., 2001).

**Measurement of mitochondrial membrane potential in cells expressing M11L constructs**

Rat2puro or Rat2M11L cells were cultured in 12 well plates ($5x10^5$ cells/well) and treated with 2 μM staurosporine for 4 h. Control cells were treated with an equivalent amount of DMSO alone. Thereafter, the cells were stained with the green fluorescent dye 3,3'dihexyloxacarbocyanine iodide ($DiOC_6$, Molecular Probes). The fluorescence intensity of this dye has been found to correlate with the mitochondrial membrane potential and provide an accurate measure of loss of this potential during apoptosis (Metivier et al., 1998). Cells were incubated with 0.5 nM $DiOC_6$ at 37°C for 10 min, harvested with trypsin and then washed and resuspended in RPMI medium lacking phenol red (Gibco Life Technologies). To verify that a decrease in the fluorescence signal intensity did accompany a loss of mitochondrial membrane potential, control cells were treated with the protonophore carbonyl cyanide m-chlorophenyl hydrazone (CCCP, Molecular Probes) that causes dissipation of the proton gradient across the mitochondria inner membrane (Metivier et al., 1998). CCCP was added to the cell culture medium at a final concentration of 50 μM during and after dye addition. Flow cytometric analysis was conducted using a Beckton-Dickinson FACSCalibur flow cytometer equipped with an argon-ion laser with 15mW of excitation at 488nm. Data were acquired at 10000 cells per sample and the fluorescent signal due to excitation of $DiOC_6$ at 488nm was detected through the FL1 channel equipped with a 530nm filter (30nm

band pass). Light scatter signals were acquired at linear gain and fluorescence signals were acquired at logarithmic gain.

# Chapter 3[3]

## M11L localizes to the mitochondrial outer membrane

[3] The data included in this chapter have been published (Everett et al., 2000) or are submitted in: The Myxoma poxvirus protein, M11L, prevents apoptosis by direct interaction with the mitochondrial permeability transition pore. Helen Everett, Michele Barry, Siow Fong Lee, Christine Frantz, Luc G. Berthiaume, Grant McFadden and R. Chris Bleackley

## Introduction

Investigation of the anti-apoptotic properties of M11L gave the first indication that this protein can act to prevent transduction of cell death signals via the mitochondrial apoptotic control point. Bcl-2 also impacts this control point in part because this protein is targeted to the outer mitochondrial membrane by means of a C-terminal transmembrane domain (Nguyen et al., 1993), a domain that is important for anti-apoptotic function (Nguyen et al., 1994; Tanaka et al., 1993). A similar correlation between mitochondrial localization and anti-apoptotic function is observed with other Bcl-2 related proteins such as Bcl-$X_L$ (Wolter et al., 1997) and the Epstein-Barr virus protein, BHRF-1 (Theodorakis et al., 1996; Yasuda et al., 1998).

Many questions surround the subject of targeting of proteins to the outer mitochondrial membrane, however two types of targeting sequence have been identified according to whether they are N- or C-terminal. Both types of outer membrane targeting motif serve as membrane anchoring sequences and are responsible for the targeting and retention of the protein in question. N-terminal membrane anchoring sequences typically contain a 'pre-sequence' that is proximal to the beginning of the protein and is followed by a transmembrane domain (Millar and Shore, 1996). Pre-sequences are found in many classical mitochondrial targeting signals and are characteristically situated at the extreme N-terminus of the protein, contain abundant positive charges and form amphipathic helices in hydrophobic environments (reviewed in (Neupert, 1997)). It is thought that the pre-sequences of N-terminal membrane anchoring sequences initiate the insertion of proteins into the outer membrane and that the transmembrane regions serve as 'stop-transfer' signals (Millar and Shore, 1996). Less is known about C-terminal membrane anchoring sequences

except that they contain a transmembrane domain (Millar and Shore, 1996) and may require a positive charge at the extreme the C-terminus (Isenmann et al., 1998).

Bcl-2 has a C-terminal membrane anchoring sequence. *In vitro* import experiments have revealed that this sequence directs Bcl-2 to the outer mitochondrial membrane in a manner that is dependent on temperature and cytosolic ATP, but independent of mitochondrial ATP and $\Delta\Psi m$. There may also be some requirement for other protein components in the outer membrane (Millar and Shore, 1996; Nguyen et al., 1993) that are not protease sensitive (Janiak et al., 1994). In contrast, association of Bcl-2 with microsomes occurs to a much lesser extent and in a mechanistically different manner in that it is dependent on cytosolic ATP and temperature but does not require other membrane components. Bcl-2 is orientated so that the C-terminus is membrane-associated and the bulk of the protein is orientated towards the cytoplasm and available for interaction with other cytoplasmic proteins.

Initial sequence analysis of M11L predicted the presence of a C-terminal transmembrane domain (Graham et al., 1992), raising the possibility that this region could serve as a signal-anchor sequence and direct the protein to intercellular membranes in a manner analogous to Bcl-2. However, the transmembrane region of M11L does not have a very pronounced hydrophobic character (Graham et al., 1992) suggesting that post-translational modification might be required to assist in anchoring of the protein. M11L was predicted not to undergo glycosylation (Graham et al., 1992) and preliminary in vitro kinase experiments did not indicate modification by phosphorylation (H. Everett, unpublished observations). The possibility remained that M11L could be modified by a fatty acid moiety in order to facilitate membrane retention. In total, information regarding the intracellular targeting and post-translational modification of

M11L was lacking, although these characteristics are known to be important for the function of Bcl-2 family members. Therefore the intracellular targeting and post-translational modification of M11L was investigated in order to better understand the mode of action of this protein.

## Results

### M11L localizes to mitochondria in infected cells

In order to study the intracellular localization of M11L, BGMK cells were infected with M11L-expressing myxoma virus (vMyxlac) or the M11L knockout virus (vMyxM11L$^-$) and 20 h post-infection, M11L was visualized by indirect immunofluorescence and confocal microscopy. As expected, M11L was detected in vMyxlac-infected cells (Figure 3.1, Panel a) and not in cells infected by the knockout virus (Figure 3.1, Panel d). In addition, M11L was observed to have a punctate, cytoplasmic distribution reminiscent of mitochondrial targeting. To determine whether M11L did, indeed, localize to mitochondria, cells were co-stained with the mitochondrion-specific dye Mitotracker Red (Figure 3.1, Panels b and e). When the fluorescent signals arising from M11L and Mitotracker Red staining in the same cells were superimposed, a uniform yellow image was produced, indicating that the two signals were co-incident (Figure 3.1, Panel c). No such signal was observed in the case of cells infected with the knockout virus (Figure 3.1, Panel f). This result provides evidence that, within myxoma virus-infected cells, intracellular M11L localizes predominantly to mitochondria.

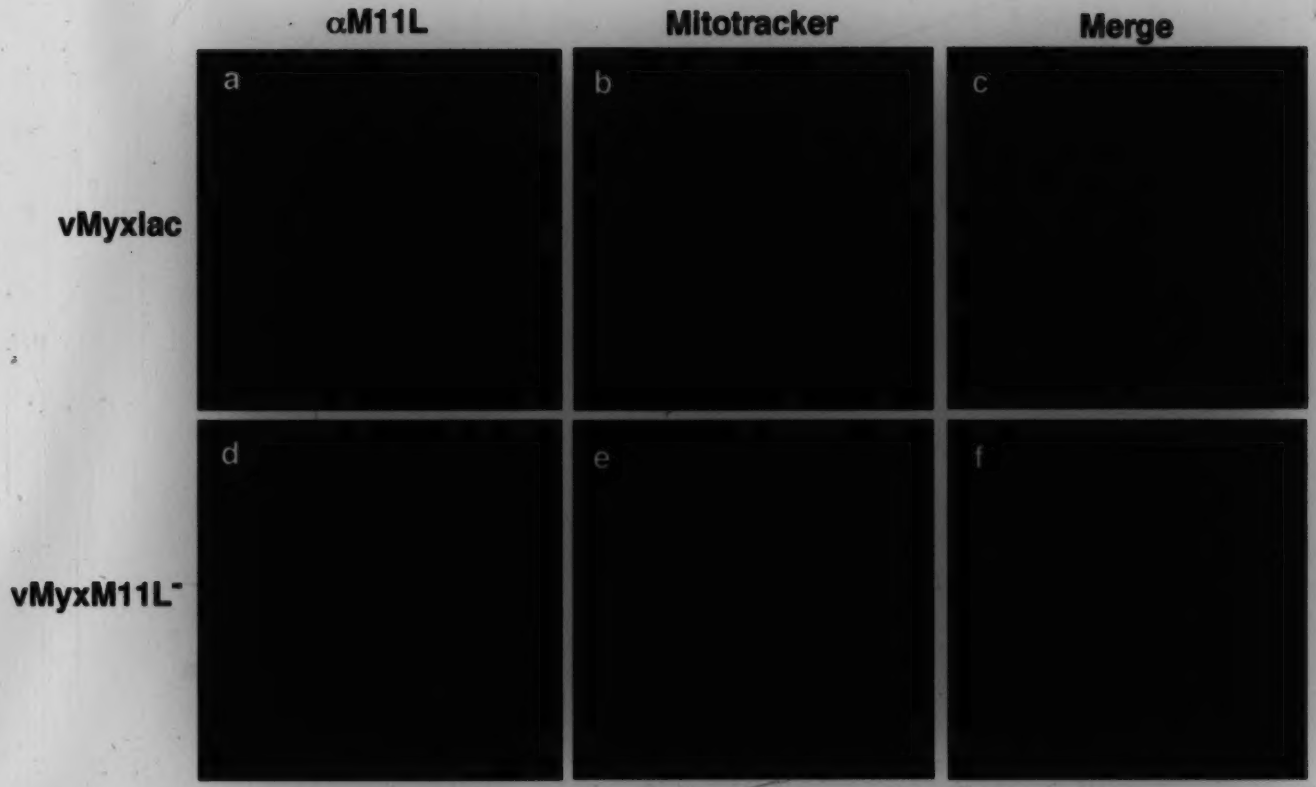


**Figure 3.1 M11L localizes to mitochondria in infected cells.** BGMK cells infected with vMyxlac or vMyxM11L⁻ were stained with Mitotracker red, fixed and then M11L was detected by indirect immunofluorescence. Confocal microscopy revealed that M11L is present in (a) vMyxlac- but not (d) vMyxM11L⁻-infected cells. Mitotracker red produced punctate mitochondrial staining (b and e), that was co-incident with the M11L signal (c). This was not observed in vMyxM11L⁻-infected cells (f). The scale bar represents 10 μm.

**Transiently expressed M11L localizes to mitochondria**

It was next important to determine whether M11L maintained this intracellular distribution when expressed in live, transfected cells and independently of other viral proteins. The GFP-expressing plasmid pS65T or pS65T-M11L, a plasmid that encodes a chimeric protein consisting of GFP appended with M11L at the C-terminus, were used to transfect COS-7 cells. GFP alone produced a diffuse signal throughout COS-7 cells (Figure 3.2, Panels a and c) reflecting its lack of intracellular targeting and lack of correspondence with Mitotracker Red staining (Figure 3.2, Panel b). The GFP-M11L fusion protein, however, displayed a punctate cytoplasmic distribution both in COS-7 cells (Figure 3.2, Panel d) and HeLa cells (data not shown) which was suggestive of association with intracellular membranes. Upon comparison of the GFP-M11L fluorescence pattern with that of Mitotracker Red in the same cells (Figure 3.2, Panel e), the two signals were found to be co-incident (Figure 3.2, Panel f) indicating that GFP-M11L localizes to mitochondria. Similarly, a mutant form of M11L designed so that the cysteine in position 141 is replaced with serine (discussed later in this chapter) also had a mitochondrial distribution (Figure 3.2 Panels g-i).

These results show that M11L, even when expressed away from the context of viral infection, is targeted to mitochondria and contains all of the sequence information necessary for correct targeting. These experiments also show that a GFP tag, when appended to the N terminus of M11L, does not alter the mitochondrial localization of the protein, and that this localization is consistent in cells having different species of origin.

**Figure 3.2 GFP-M11L localizes to mitochondria.** COS-7 cells expressing (a) GFP, (d) GFP-M11L or (g) GPF-C141S were co-stained with Mitotracker red (b, e and h) and visualized by confocal microscopy. The merged images show that GFP alone is distributed throughout the cytoplasm and is not targeted (c). In contrast, GFP-M11L and GFP-C141S, a construct encoding a variant form of M11L, co-localize with the mitochondrial marker (f and i). The scale bar represents 10 μm.

**M11L is present on the mitochondrial outer membrane**

To investigate the topology of M11L within mitochondria, experiments were conducted to determine whether membrane-associated M11L is proteinase K sensitive and therefore exposed to the cytoplasm. For these experiments, the human hepatocyte-derived HepG2 cell line was used as the cytosol of these cells contains a large proportion of mitochondria. In order to express high concentrations of M11L, HepG2 cells were infected with an M11L over-expressing vaccinia virus (VVM11L). Parallel infection with the vaccinia virus VV601, which does not express M11L, was conducted for control purposes. Following infection for 12h, digitonin extracts were prepared and separated into pellet and supernatant fractions. Duplicate samples were either treated with Proteinase K or left untreated prior to analysis of immunoprecipitated M11L. COX IV, a protein that resides in the inner mitochondrial membrane, was analyzed in parallel for control purposes.

As can be seen from Figure 3.3 (upper panel), the majority of the M11L protein was present in the pellet fraction (Lanes 1 and 5) which is enriched for membranous structures. A small proportion of M11L could be detected in the soluble fraction (Lanes 3 and 7) as would be expected for a protein which is produced by a virus with a cytoplasmic site of replication. M11L protein was not present in cells infected with the control virus, as expected (Lanes 2, 4, 6 and 8). COX IV was only detected in the pellet fractions (Figure 3.3, Lower Panel, Lanes 1, 2, 5 and 6). When the protease sensitivity of M11L was assessed, it was evident that M11L present in the pellet fraction was Proteinase K sensitive (Figure 3.3, Upper Panel, Lane 1). M11L present in the supernatant fraction was also protease sensitive, as revealed by over-exposure of the immunoblot (data not shown). In contrast, COX IV, present in the pellet fraction, was protease resistant (Figure 3.3, Lower Panel, Lanes 1 and 2). These data provide

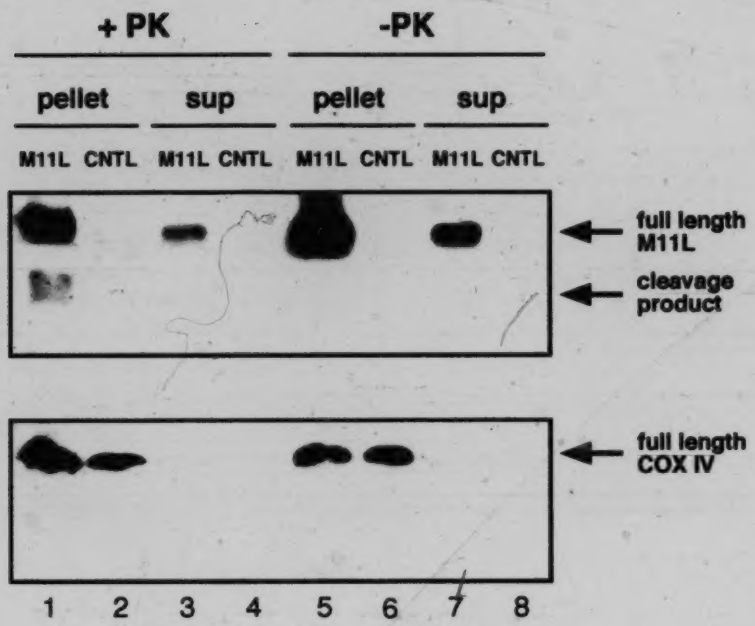


**Figure 3.3 Membrane-associated M11L is protease sensitive.** HepG2 cells infected with M11L-expressing VVM11L (M11L) or a control vaccinia virus (CNTL) were lysed in a digitonin buffer and pellet (pellet) and supernatant (sup) fractions were isolated. Replicate samples were treated with proteinase K (PK) or left untreated prior to SDS-PAGE. Immunoblot analysis of immunoprecipitatedM11L (top panel) and COX IV in the pellet fraction of cell lysates (bottom panel)revealed that only M11L was protease sensitive.

evidence that M11L, although associated with mitochondria, is exposed on the cytoplasmic face of these organelles.

**M11L is palmitoylated**

Although M11L has a putative C-terminal transmembrane domain (Figure 1.5), this region does not have a notable hydrophobic character. This raised the possibility that fatty acid modification might assist in the anchoring of M11L to the mitochondrial outer membrane. M11L did not have consensus sequences for modification by myristoyl or prenyl moieties, therefore palmitoylation remained as the most likely fatty acid modification, if any (Resh, 1999).

To test whether M1lL is palmitoylated, BGMK cells were infected with the M11L-expressing vMyxlac, or with the M11L knockout virus, vMyxM11L. The infected cells were then incubated for 18 h in medium containing the radiolabelled fatty acid derivative $^{125}$I iodo-palmitate (Berthiaume et al., 1995). M11L was detected by immunoprecipitation followed by SDS-PAGE and autoradiography or immunoblotting. As shown in Figure 3.4, M11L was specifically immunoprecipitated from cells infected with vMyxlac (Upper panel, Lane 1) but not from cells infected with the knockout virus (Upper panel, Lane 3). M11L was also not immunoprecipitated when control rabbit serum was used (Upper Panel, lanes 2 and 4). When autoradiography was performed, a signal corresponding to radiolabelled M11L was detected (Lower panel, Lane 1). The thioester bond whereby palmitate becomes covalently linked to a cysteine side chain is labile when exposed to neutral hydroxylamine treatment. Treatment of the radiolabelled M11L protein with neutral hydroxylamine attenuated the $^{125}$I iodo-palmitate signal (data not shown) supporting the idea that palmitate is indeed attached to M11L by means of a labile thioester bond. These results suggested that M11L undergoes

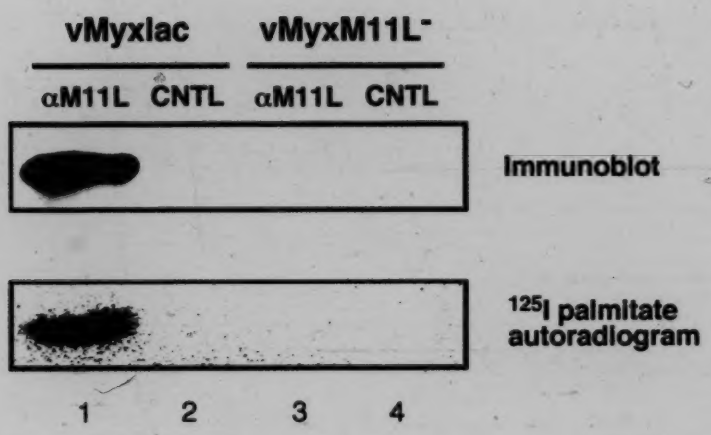


**Figure 3.4 M11L is palmitoylated.** BGMK cells infected with vMyxlac or vMyxM11L⁻ were labeled with $^{125}$I palmitate. Proteins were immunoprecipitated using an anti-M11L antibody (αM11L) or control (CNTL) antibody and analysed by SDS-PAGE and immunoblotting (upper panel) or autoradiography (lower panel).

palmitoylation, a post-translational modification involving covalent attachment of the fatty acid palmitate.

**M11L is palmitoylated on cysteine 141**

Palmitate is characteristically added to cysteine side chains by a reversible thioester linkage (Resh, 1999). Viral proteins that undergo palmitoylation are frequently modified on cysteine residues that are adjacent to or within hydrophobic domains (Veit et al., 1996). Therefore, the cysteine residue at position 141 in the M11L sequence was most likely to be modified by palmitoylation as C141 is immediately adjacent to the hydrophobic mitochondrial targeting sequence. To test the prediction that C141 is palmitoylated, the *M11L* gene sequence was altered to introduce a point mutation and permit the expression of a variant protein, designated C141S, in which the cysteine at position 141 was replaced by serine. The *M11L* and *C141S* genes were cloned into the poxvirus expression vector pMJ602 (Davison and Moss, 1990) downstream of the poxvirus synthetic late promoter,

The palmitoylation status of the wild-type M11L and C141S proteins was compared using an approach previously used to study palmitoylated vaccinia virus proteins (Grosenbach et al., 1997). BGMK cells were infected with vaccinia virus only or infected with vaccinia virus and simultaneously transfected with the plasmids pMJM11L or pMJC141S to allow the expression of wild type M11L or the C141S mutant. Control cells were transfected with the empty pMJ602 vector. Cells were then labeled with $^{125}$I iodo-palmitate and M11L was immunoprecipitated. Samples were analyzed by SDS-PAGE and autoradiography or immunoblotting. As can be seen from Figure 3.5 (upper panel), immunoblot analysis revealed that, as expected, wild type and mutant M11L were produced using this infection-transfection approach (Lanes 3 and 5). However,

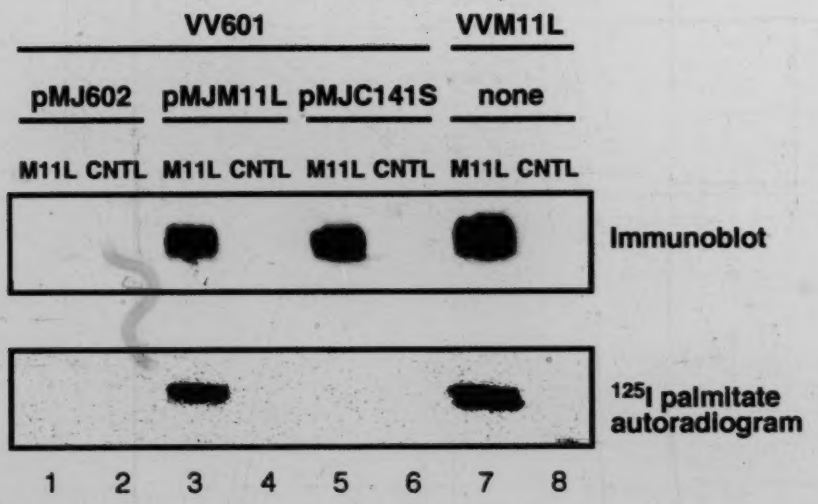


**Figure 3.5 M11L is palmitoylated on cysteine 141.** Cells were infected with vaccinia virus (VV601) alone or infected with vaccinia virus and transfected with plasmids pMJM11L or pMJC141S to allow expression of wild-type M11L or the C141S variant respectively. Cells were also infected with control VVM11L. All monolayers were labeled with $^{125}$I-palmitate and samples were immunoprecipitated with anti-M11L (M11L) or control (CNTL) antibody. Immunoblot analysis revealed that wild type and mutant M11L was expressed (upper panel). Autoradiography (lower panel) revealed that a signal was produced by wild-type M11L but not the C141S point mutant indicating that the C141S mutant is not palmitoylated.

approximately five-fold more of the C141S mutant sample needed to be analyzed in order for equal amounts of protein to be detected on the immunoblot. M11L was also produced in cells infected with the M11L-expressing vaccinia virus VVM11L, as expected (Lane 7) and was not present in control samples (Lanes 1, 2, 4, 6 and 8). However, autoradiography revealed that an $^{125}I$ signal corresponding only to wild-type M11L (Lower Panel, Lanes 3 and 7) and not to the C141S mutant (Lower Panel, Lane 5) could be detected. This result provides evidence that wild type M11L is palmitoylated on cysteine 141, and that mutation of this amino acid prevents the fatty acid modification of the protein. In addition the radioactive signal was considerably diminished by treatment of the SDS-PAGE analyzed samples with neutral hydroxylamine, but not by Tris-HCl at the same pH (data not shown), supporting the idea that the label was attached to the protein by means of a labile thioester linkage. It can be concluded from these experiments that M11L is modified by the reversible addition of palmitate and that this modification occurs on cysteine 141.

**M11L contains a C-terminal mitochondrial targeting signal**

Since the C terminal 24 amino acids of M11L include a stretch of 18 amino acids that constitute a putative transmembrane domain, it was possible that this domain was important for targeting. To address this question, the localization of a GFP-tagged, truncated form of M11L lacking the C-terminal 24 amino acids was investigated. When visualized in live COS-7 cells M11L displayed characteristic punctate distribution (Figure 3.6 panel a) and co-localization with the Mitotracker red mitochondrial marker (Figure 3.6 panel b and c). In contrast, the truncated form of M11L was distributed diffusely throughout the cytoplasm and nucleus in COS-7 cells (Figure 3.6, panel d) and showed no correspondence with the staining pattern due to Mitotracker Red (Figure 3.6, Panels e and f). The same

**Figure 3.6 M11L contains a mitochondrial targeting signal.** EGFP appended with wild-type M11L, a truncation mutant (stop) or the mitochondrial targeting sequence (mt) (panels a,d,g) was visualized in live COS-7 cells. Cells were co-stained with Mitotracker red (panels b,e,h). The EGFP-M11L and EGFP-mt signals were co-incident with Mitotracker (c, i) but the EGFP-stop signal was not (f). Scale bar represents 10 μm. mt=KISVYLTAAVVGFVAYGILKWYRGT

result was obtained using HeLa cells (data not shown). This shows that the C-terminal 24 amino acids are necessary for M11L targeting to mitochondria, and removal of this domain, which includes the putative transmembrane region and short 5 amino acid positively charged tail, prevents mitochondrial localization. Our conclusion was reinforced by the finding that a related mutant, constructed to replace the terminal 16 amino acids (consisting of 10 residues of the transmembrane region and the C terminal tail) with 9 irrelevant amino acids, also failed to localize to mitochondria (data not shown).

We next sought to determine whether the 25 amino acid C-terminal region of M11L alone was sufficient for mitochondrial targeting. The coding sequence for this minimal region (designated mt, Figure 3.6) was appended to GFP and was found to direct GFP to punctate cytoplasmic structures in both COS-7 cells (Figure 3.6, Panel g) and in HeLa cells (data not shown). When the distribution of this fluorescent signal was compared to that of Mitotracker Red (Figure 3.6, panel h) in the same cells, the two signals were found to be co-incident (Figure 3.6, panel i).

Hence, the C-terminal 25 amino acids of M11L comprise a signal that is sufficient for mitochondrial targeting. This targeting motif includes an 18 amino acid putative transmembrane domain flanked by positively charged lysine residues adjacent to a short 6 amino acid C-terminal tail with a net positive charge (Figure 3.7). A truncated form of this sequence consisting of only the last 19 amino acids of M11L failed to localize GFP to mitochondria (data not shown), indicating the requirement for a hydrophobic stretch sufficiently long to form a transmembrane segment within the targeting signal.

The M11L C-terminal targeting signal has properties of a signal-anchor targeting sequence and contains distinct topological features. These

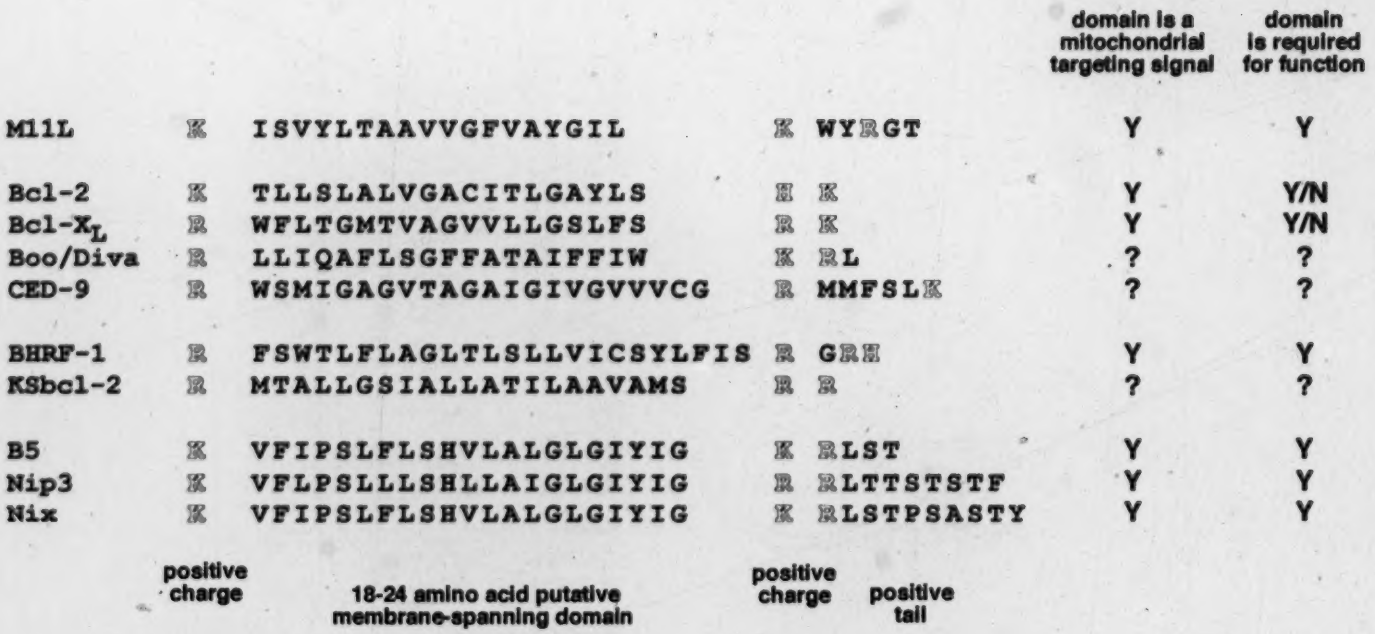
domain is a mitochondrial targeting signal
domain is required for function
M11L K ISVYLTAAVVGFVAYGIL K WYRGT Y Y
Bcl-2 K TLLSLALVGACITLGAYLS H K Y Y/N
Bcl-XL R WFLTGMTVAGVVLLGSLFS R K Y Y/N
Boo/Diva R LLIQAFLSGFFATAIFFIW K RL ? ?
CED-9 R WSMIGAGVTAGAIGIVGVVVCG R MMFSLK ? ?
BHRF-1 R FSWTLFLAGLTLSLLVICSYLFIS R GRH Y Y
KSbcl-2 R MTALLGSIALLATILAAVAMS R R ? ?
B5 K VFIPSLFLSHVLALGLGIYIG K RLST Y Y
Nip3 K VFLPSLLLSHLLAIGLGIYIG R RLTTSTSTF Y Y
Nix K VFIPSLFLSHVLALGLGIYIG K RLSTPSASTY Y Y
positive charge
18-24 amino acid putative membrane-spanning domain
positive charge
positive tail

**Figure 3.7 Consensus targeting sequences from M11L and other apoptotic modulators.** These C-terminal motifs can be identified in the anti-apoptotic cellular Bcl-2 proteins, Bcl-2, Bcl-$X_L$, Boo/Diva and Ced-9, the viral anti-apoptotic proteins, M11L, BHRF-1 and KSBcl-2 as well as in several cellular proteins with distant homology to Bcl-2, the anti-apoptotic B5 protein and pro-apoptotic Nip3 and Nix proteins.

features include a hydrophobic region flanked by positively charged residues and a short, positively charged tail at the extreme C terminus. A similar targeting motif was found to target Vesicle-associated membrane protein 1B (VAMP-1B) and Bcl-2 (Isenmann et al., 1998). However, this targeting signal is potentially much more wide spread and is present in diverse Bcl-2 family members (Figure 3.7).

**The M11L mutant R164G has a distinct subcellular localization**

Based on published studies (Isenmann et al., 1998) it was anticipated that the positive tail within the targeting sequence would be of functional importance. Accordingly, an R164G mutant was generated that encodes a protein in which the positively charged arginine residue at position 164 has been replaced by a neutral glycine residue, thereby removing the net positive charge of the C terminal tail in the targeting motif. The effect of the R164G mutation on targeting was investigated by expression of EGFP appended with the wild type mitochondrial targeting sequence (EGFP-mt), or appended with the targeting sequence incorporating glycine at position 164 (EGFP-mtR164G). HeLa cells expressing these constructs were stained with the mitochondrion-specific dye, Mitotracker red, and visualized by confocal microscopy (Figure 3.8). Whereas the EGFP-mt (Panel a) and Mitotracker red (Panel b) signals were co-incident (Panel c), the EGFP-mtR164G (Panel d) and Mitotracker red (Panel e) signals were not (Panel f), indicating that removal of the C-terminal positive charge of the M11L targeting signal disrupts the mitochondrial targeting function. The same result was observed in Cos-7 cells (data not shown). When the full-length M11L protein containing the R164G substitution was appended to EGFP (EGFP-R164G), it was found to have the same distribution pattern as the EGFP-mtR164G chimera and both appeared to localize to intracellular membranes. When the EGFP-R164G fluorescent signal (Panel g) was compared to that of the endoplasmic reticulum-resident protein,

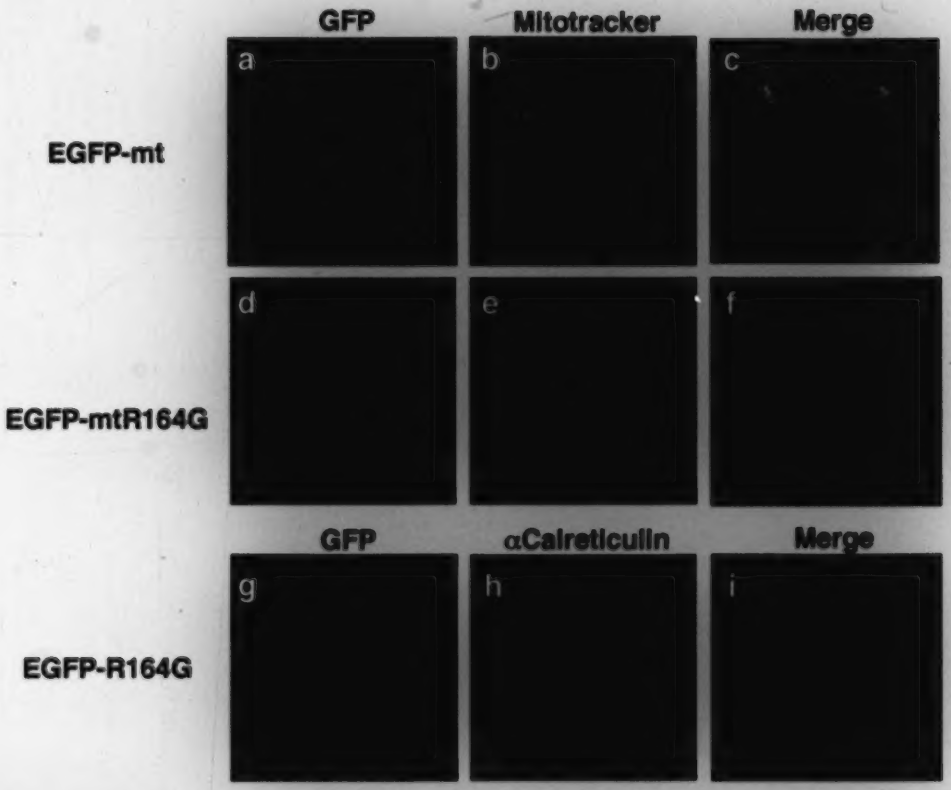


**Figure 3.8 The R164G variant of M11Lhas a distinct subcellular localization.** Representative confocal micrographs of HeLa cells expressing EGFP constructs (panels a,d and g) and co-stained with Mitotracker red (panels b and e) or the endoplasmic reticulum marker, calreticulin (h). EGFP appended to the wild-type M11L targeting sequence (EGFP-mt) co-localized with Mitotracker (panel c) whereas EGFP appended by the mutant sequence (EGFP-R164G) did not (panel f). The full length R164G mutant protein directed EGFP to intracellular membranes, and partially co-localized with calreticulin (panel i). Scale bar represents 10 μm.
**mt = KISVYLTAAVVGFVAYGILKWYRGT**

calreticulin (Panel h), co-incident signals were observed (Panel i), indicating that a proportion of the R164G variant protein localizes to the endoplasmic reticulum.

## Discussion

The studies described in this chapter investigated the localization of wild-type M11L expressed in the context of myxoma virus infection as well as a GFP-M11L chimera visualized in live, transfected cells. Both approaches demonstrated that M11L is primarily targeted to mitochondria. A previous study (Graham et al., 1992) based on indirect immunofluorescence analysis of non-permeabilized cells, revealed that M11L could be detected on the surface of infected cells. However, surface M11L probably represents an extremely minor proportion of the total amount of protein produced in myxoma virus-infected cells. Membrane-associated M11L remains accessible to proteinase K digestion indicating that this protein is associated with the outer mitochondrial membrane and is oriented towards the cytoplasm. Hence, M11L has an intracellular distribution similar to many Bcl-2-like anti-apoptotic proteins of both cellular and viral origin (Gross et al., 1999; Vander Heiden and Thompson, 1999).

M11L, unlike the C141S mutant, is modified by the fatty acid palmitate added to cysteine at position 141, immediately adjacent to the hydrophobic mitochondrial targeting signal. It is interesting that the M11L homolog encoded by Rabbit fibroma virus has a short, in frame deletion that effectively removes this cysteine from this isoform of M11L ((Graham et al., 1992) and Figure 1.5). However, little is known about the function of the Rabbit Fibroma virus M11L isoform. M11L can be classified as a type I palmitoylprotein (Resh, 1999). Other palmitoylated viral proteins also have this classification and include the p37 envelope protein of vaccinia virus (Grosenbach et al., 1997), gp160 of HIV-1 (Rousso et al., 2000), the Adenovirus Death Protein (ADP) (Hausmann et al., 1998) and the $M_2$ channel protein of influenza virus (Holsinger et al., 1995). The reversible

modification of proteins by the fatty acid palmitate can have varied functional consequences and affect protein localization and/or activity (Resh, 1999). For example, palmitoylation is involved in regulating the activity of $\beta_{2a}$ subunits of calcium channels (Hurley et al., 2000). Palmitoylation can also play a functional role within viral proteins. Expression of palmitoylation-defective p37 protein by a recombinant vaccinia virus results in a protein that fails to target correctly to the membranes of the *trans*-Golgi network and viral particles that are unable to bud from the cell (Grosenbach and Hruby, 1998). Palmitoylation-defective gp160 of HIV does not correctly associate with lipid rafts, is not efficiently packaged into virions and, when expressed by a recombinant HIV strain, results in the production of viral particles with reduced infectivity (Rousso et al., 2000). However, the presence or absence of palmitate on other viral proteins, such as $M_2$ or ADP has no currently identifiable functional consequences (Hausmann et al., 1998; Holsinger et al., 1995).

In addition, the non-palmitoylated C141S mutant has a sub-cellular localization indistinguishable from that of wild-type M11L. However, immunoblot analysis revealed that levels of C141S mutant were reduced compared to the levels of M11L in infected-transfected cells. This may imply that palmitoylation can modulate the localization and/or degradation of M11L.and the effects of the C141S mutation on protein anchoring and/or protein turnover remain to be determined.

M11L incorporates a C-terminal 25 amino acid motif that is necessary and sufficient for mitochondrial targeting. Interestingly the motif excludes the palmitoylated cysteine, indicating that fatty acid modification is not required for targeting. A C-terminal transmembrane region associated with positive charges that is required for mitochondrial targeting has been previously identified in VAMP-1 and Bcl-2 (Isenmann et al., 1998). Based

on these experimental findings, combined with the features identified as a result of investigating the targeting of M11L, a more defined consensus C-terminal mitochondrial targeting sequence can be proposed. This sequence is suggested to include characteristic topological features, namely a hydrophobic transmembrane region flanked by positively charged residues which is adjacent to a positively charged tail (Everett et al., 2000). Interestingly, C-terminal targeting motifs responsible for directing other proteins involved in apoptosis to the outer mitochondrial membrane are also found to conform to this consensus (Figure 3.7). Included in this category are Bcl-2 (Nguyen et al., 1994; Tanaka et al., 1993), Bcl-$X_L$ (Wolter et al., 1997), BHRF-1 (Theodorakis et al., 1996; Yasuda et al., 1998), B5 (Ohi et al., 1999), Nip3 (Chen et al., 1997; Yasuda et al., 1998) and Nix (Chen et al., 1999). This motif is also present in the Bcl-2 family members Boo/Diva (Inohara et al., 1998; Song et al., 1999), CED-9 (Wu et al., 1997) and KSBcl-2 from HHV8 (Cheng et al., 1997), although the intracellular localization of these proteins and/or the precise role of this motif in targeting is unknown at present.

It is recognized that arginine or lysine patches occur at the cytoplasmic ends of plasma membrane receptors and that these positively charged regions serve to anchor the receptor to the negatively charged phospholipids of the membrane surface (Von Heijne, 1992). In addition, positively charged N-terminal presequences within mitochondrial targeting domains of proteins have been shown to preferentially accumulate at the negative surface of the mitochondrial outer membrane (Swanson and Roise, 1992). Previous studies (Isenmann et al., 1998) had also indicated that a C-terminal positive charge is important for mitochondrial targeting. Based on these observations and the elements identified in the consensus targeting sequence, it was predicted that alteration of the positive at the C-terminus of M11L would perturb the localization. Indeed, the R164G variant of M11L, which has a

neutral C-terminus, was found to have an altered subcellular localization and, although still directed to intracellular membranes, was directed mainly to the endoplasmic reticulum. It is possible that this R164G mutant becomes localized to any hydrophobic surface by default. This would imply that the positive tail is confers the mitochondrial targeting specificity to the sequence. It was observed that mutations that reduced the length of the membrane-spanning domain also prevented membrane targeting, indicating that the transmembrane domain is also an important element for targeting. In addition, mutations that increased the length of the C-terminal tail abolished the function and targeting of M11L (Opgenorth et al., 1992) and unpublished observations). This perhaps reflects the need for a tail that can translocate or 'flip' across a lipid bilayer with minimal energy requirements.

## Materials and Methods

### Cells

BGMK monkey kidney cells (obtained from Dr. S. Dales, University of Western Ontario) were maintained in DMEM (Gibco BRL) supplemented with 10% newborn calf serum (Gibco BRL). HepG2 human hepatocellular carcinoma cells (ATCC) and COS-7 monkey fibroblasts were maintained in DMEM supplemented with 10% FBS. HeLa human cervical carcinoma cells (ATCC) were cultured in RPMI medium (Gibco BRL) supplemented with 10% FBS. All media contained 200 U/ml penicillin and 200 µg/ml streptomycin (Gibco BRL).

**Virus infections**

BGMK cells were infected with the recombinant myxoma viruses vMyxlac (described in Chapter 2) or vMyxM11L⁻, a recombinant myxoma virus in which the *M11L* gene has been selectively disrupted by targeted insertion of the *Escherichia coli lacZ* gene under the control of the vaccinia virus synthetic late promotor (Opgenorth et al., 1992). HepG2 and COS-7 cells were infected with VVM11L, a recombinant vaccinia virus that over-expresses M11L under the control of the vaccinia virus synthetic late promotor (Graham et al., 1992) or a control vaccinia virus, VV601. In all cases, 1x $10^6$ cells were infected with virus at an MOI of 10 at 37°C for 1 h in a total volume of 0.5 ml medium before being replenished with 2 ml medium for overnight incubation.

**Confocal microscopy of infected cells**

In order to study M11L localization, BGMK cells (1x$10^5$) grown in 35mm cell culture dishes (Corning) containing glass number 1 coverslips (Fischer Scientific) were infected with either vMyxlac or vMyxM11L⁻ and were treated 20 h post-infection with the mitochondria-specific fluorescent marker Mitotracker Red CXMRos (Molecular Probes) at a final concentration of 30 ng/ml. Accumulation of the dye was allowed to proceed for 20 min at 37°C. The cells were fixed with 2% paraformaldehyde/PBS for 30 min at room temperature and permeabilized for 2 min with cold 0.1% Triton X-100/ 0.1% sodium citrate buffer. Cells adhering to coverslips were then incubated for 20 min at room temperature with a polyclonal rabbit anti-M11L antibody (Graham et al., 1992) diluted 1:50 in PBS followed by incubation for 20 min at room temperature with a secondary FITC-conjugated anti-rabbit antibody (Jackson ImmunoResearch Laboratories) at a dilution of 1:200 in PBS. Coverslips were mounted using 50% PBS/ 50% glycerol solution and

confocal images were obtained using a LSM510 Laser scanning confocal microscope mounted on a Zeiss Axiovert 100M microscope equipped with a 63x 1.4 oil immersion Plan-Apochromat objective. FITC excitation was induced by illumination at 488nm and the fluorescent signal was collected using a 505-530nm band pass filter. Mitotracker Red fluorescence was induced by illumination at 543nm and was detected using a 560nm long pass filter.

**Analysis of Protease sensitivity**

HepG2 cells ($5x10^5$) were infected VVM11L or VV601 and 12 h post-infection the cells were trypsinized, washed with PBS and resuspended in 200 µl digitonin lysis buffer (75 mM NaCl, 1 mM $NaH_2PO_4$, 8 mM $Na_2HPO_4$, 250 mM sucrose and 95 µg/ml digitonin) at 4°C for 5 min. This treatment selectively permeabilizes the plasma membrane without disrupting membranes comprising intracellular organelles (Nguyen et al., 1993). Both samples were divided into four aliquots of 50 µl each and centrifuged at 15000g for 15 min at 4°C and the supernatants were retained. The pellets were resuspended in 50 µl digitonin lysis buffer. Duplicate samples were then treated with the protease inhibitor phenylmethylsulfonyl fluoride (PMSF, Sigma) at a final concentration of 1mM immediately or treated with 2.5 µg/µl proteinase K (Boehringer Mannheim) at 4°C for 20 min before the reaction was quenched by PMSF addition. One of the duplicate samples was then resuspended in SDS sample buffer and the whole cell lysate was used to detect cytochrome c oxidase subunit IV (COX IV). The other sample was used to immunoprecipitate M11L as described in Chapter 2. For COX IV and M11L detection, samples were subjected to SDS-PAGE on a 15% gel and electroblotted onto Immobilon-P membrane (Millipore). COX IV was detected using a primary mouse monoclonal antibody (Molecular Probes) at a concentration of 0.4 µg/ml and a secondary anti-mouse-HRP

conjugate (Bio-Rad) at 1:6000 dilution followed by ECL detection (Amersham). M11L was detected using the primary rabbit polyclonal antibody (Graham et al., 1992) at a dilution of 1:500 and a secondary Protein A-HRP conjugate (Pierce) at a dilution of 1:10000, followed by ECL detection.

**Infection-transfection and palmitoylation studies**

BGMK cells ($2.5 \times 10^6$) were infected with vMyxlac or vMyxM11L for 4h The medium was then replenished with serum-free DMEM containing 25 μCi iodo-palmitate (Berthiaume et al., 1995) and the infection was allowed to proceed for a further 18 h. The medium was then aspirated, the cells were lysed directly and M11L was immunoprecipitated as described in Chapter 2. Samples were diluted in SDS loading buffer containing a reduced (2 mM) concentration of dithiothreitol to avoid hydrolysis of thioester bonds linking labeled palmitate to proteins and then analyzed by SDS-PAGE followed by autoradiography or immunoblotting with an anti-M11L antibody.

In order to generate a coding sequence to direct expression of an M11L point mutant in which the cysteine in position 141 was replaced by serine, a mutation was introduced into the *M11L* gene using the Altered sites II *in vitro* mutagenesis system (Promega) and the oligonucleotide (GTAGCGGCGGGTCGAAAATATCCGTC). Both wild type *M11L* and the *C141S* variant genes were cloned into the poxvirus expression vector pMJ602 (Davison and Moss, 1990) to produce the plasmids pMJM11L or pMJC141S. This was to allow expression of M11L or the C141S variant in vaccinia virus-infected cells under the control of the vaccinia virus synthetic late promoter.

The palmitoylation status of M11L was investigated using an approach described previously (Grosenbach et al., 1997). Subconfluent BGMK cell monolayers (2.5 x $10^6$ cells) were each infected with vaccinia virus VV601 diluted in 1ml Optimem (Gibco BRL). Concurrently, the cells were transfected with 10 μg pMJM11L or pMJC141S plasmid DNA complexed with 30 μl Lipofectin (Gibco BRL) added to the viral inoculum in a total volume of 200 μl Optimem in each case. For control purposes, the vector pMJ602 was included in a parallel infection-transfection and a separate monolayer was infected with M11L-expressing VVM11L. The infection-transfection was allowed to proceed at 37°C for 4 h and the cells were replenished with Optimem supplemented with 3% FBS and 2 mM L-glutamine. After 2 h, the medium was replaced with serum-free DMEM containing 25 μCi iodo-palmitate and the incubation was continued for 24 h. M11L was detected as described above.

**GFP chimera expression plasmids**

To generate the expression construct GFP-M11L and direct the expression of a fusion protein consisting of the Green Fluorescent Protein (GFP) appended to the N terminus of M11L, the *M11L* coding sequence was excised from the pT7M11L plasmid as an *Xho*I-*Xba*I fragment and subcloned into the *Sal*I-*Xba*I sites of the GFP expression vector S65T-C1 (Clontech) encoding the S65T variant of GFP. This ensured that the *M11L* sequence was inserted downstream of and in frame with the GFP coding sequence.

The *M11L* and *C141S* genes were subcloned into the pEGFP-C1 vector (Clontech) to generate the pEGFP-M11L expression construct. This construct was designed to allow expression of M11L appended to the C terminus of the EGFP (enhanced) variant of GFP that has a greater fluorescence intensity than the S65T form of GPF. A construct encoding a

truncated variant of M11L was created using a PCR-based approach. The *M11L* coding sequence was amplified using the 5'Xho primer (GGATCTCGAGATGATGTCTCGTTTAAAGAC) and a 3' primer M11Lstop (AACTGCCGCGGTTAGATAGACGGATCATTT) incorporating a stop codon in place of the codon specifying isoleucine at position 143, the first amino acid of the hydrophobic region. An *Eco*RV-*Sac*II fragment containing the mutated codon was excised from this amplified PCR product and used to replace the corresponding *Eco*RV-*Sac*II fragment of the wild-type *M11L* gene within the pEGFP-M11L vector to generate pEGFP-stop.

In order to identify a minimal mitochondrial targeting signal contained in M11L, a *Taq*I-*Xba*I fragment containing the coding sequence for the last 25 amino acids of the protein was cloned into the *Nsp*V-*Xba*I sites within the multiple cloning site of pEGFP-C1 to generate pEGFP-mt. The coding sequence for the R164G M11L variant was generated by PCR mutagenesis using the pEGFP-mt vector as a template. A 5' primer (GGATCACTCTCGGCATGG) within the *EGFP* sequence and the 3' primer 3'R164G (CGTAGTCGACTAGGTCCCTCCGTAC) was used to amplify a C terminal region of M11L incorporating the *R164G* mutation. A restriction fragment encoding the *R164G* mutation was used to replace the equivalent fragment in the mt sequence of pEGFP-mt to generate pEGFP-mtR164G. Similarly, this fragment was used to replace the corresponding region in pEGFP-M11L to allow expression of the full length R164G point mutant appended to the C terminus of EGFP. Correct generation of each construct was verified by DNA sequencing analysis.

**Confocal microscopy of cells expressing GFP chimeric proteins**

Images of live cells were obtained by growing cells in 3.5 cm diameter cell culture dishes modified so that a section of the base was replaced by a

glass number 1 coverslip (Fischer Scientific). Partially confluent monolayers of COS-7 or HeLa cells were each transfected with 4 μg of respective pEGFP constructs using the Lipofectin Plus reagent and Optimem medium or Lipofectamine 2000 reagent (Gibco Life Technologies) according to the manufacturer's specifications. The transfected cells were incubated for 24 h and mitochondria were stained with Mitotracker Red CXMRos (Molecular Probes) by addition of the dye to the culture medium at a concentration of 15 ng/ml. The cell culture medium was then replenished with RPMI medium lacking phenol red (Gibco Life Technologies) and live cell were examined by confocal microscopy using the same filter settings described previously. Red and green signals were collected sequentially to eliminate bleed-through and neutral density filters were set at levels of 80% or higher to minimize photobleaching. To identify co-localization with the endoplasmic reticulum, cells transfected with pEGFP-R164G were fixed using 4% paraformaldehyde dissolved in PBS, treated with a primary goat polyclonal antibody directed against the endoplasmic reticulum-resident protein, calreticulin (Michalak and MacLennan, 1980) and a secondary Cy3-conjugated anti-rabbit antibody (Jackson ImmunoResearch). Fluorescent signals were visualized by confocal microscopy using the same settings as above.

# Chapter 4[4]

## M11L plays an important role in infected cells and directly modulates the permeability transition pore

[4] [4]The data included in this chapter have been published (Everett et al., 2000) or are submitted in: The Myxoma poxvirus protein, M11L, prevents apoptosis by direct interaction with the mitochondrial permeability transition pore. Helen Everett, Michele Barry, Siow Fong Lee, Christine Frantz, Luc G. Berthiaume, Grant McFadden and R. Chris Bleackley

## Introduction

As described in Chapter 1, apoptotic cell death is frequently accompanied by two marked mitochondrial changes that can occur independently, but frequently occur concurrently (reviewed in (Bernardi et al., 2001; Kroemer and Reed, 2000; Martinou and Green, 2001; Zamzami and Kroemer, 2001)). One of these changes is the release into the cytosol of numerous proteins, including apoptotic effectors and co-factors that are normally retained in the intermembrane space between the inner and outer mitochondrial membranes. The other notable mitochondrial change is the abrupt loss of the electrochemical potential ($\Delta\Psi_m$) normally maintained across the inner membrane. Either one of these two mitochondrial events could presage commitment to cell death and consequently, the exact mitochondrial perturbation required to ensure the death of a cell remains controversial.

The loss of $\Delta\Psi_m$ occurs as a result of the sudden opening of a mitochondrial megachannel referred to as the permeability transition (PT) pore. The PT pore consists of three core components: the Voltage Dependent Anion Carrier (VDAC) that resides in the outer mitochondrial membrane, the Adenine Nucleotide Transporter (ANT) in the inner membrane and cyclophilin D that is associated with the matrix surface of the ANT (Crompton, 1999). Pro- and anti-apoptotic Bcl-2 proteins of both viral and cellular origin are reported to physically associate with the ANT and VDAC subunits and to regulate the PT pore as described in Chapter 1. However the molecular mechanisms underlying the effects of these proteins are presently unclear. The PT pore incorporates several accessory proteins including Creatine Kinase, Hexokinase II and the Peripheral Benzodiazepine Receptor (PBR) (Bernardi et al., 2001; Crompton, 2000; Gavish et al., 1999). The role of these proteins in apoptosis modulation remains poorly understood.

The PBR is an 18 kDa integral membrane protein which resides in the outer mitochondrial membrane and associates both physically and functionally with the VDAC and ANT components of the PT pore (Kinnally et al., 1993; McEnery et al., 1992). The PBR has a wide distribution amongst 'peripheral-type' tissues and, within leukocyte lineages, has the highest levels in monocytes and polymorphonuclear cells (Carayon et al., 1996). Distinct pharmacological properties also characterize the PBR, which shows highest affinity for the diazepine 4'-chlorodiazepam (Ro 5-4864) and the isoquinoline carboxamide analog, PK11195 (Le Fur et al., 1983; Marangos et al., 1982). Porphyrins are the major endogenous ligands of the PBR, with the heme precursor, protoporphyrin IX (PPIX) having the strongest binding interaction (Snyder et al., 1987; Verma and Snyder, 1988; Verma and Snyder, 1987).

Mounting experimental evidence implicates the PBR in apoptosis modulation. Firstly, PBR over-expression in hematopoeitic cells correlates with resistance to $H_2O_2$-mediated cell death (Carayon et al., 1996). Secondly, PBR ligands can induce apoptosis, presumably by binding PBR and interfering with the function of the PT pore. In particular, exogenous addition of PPIX (Marchetti et al., 1996) or PK11195 (Fennel et al., 2001; Fischer et al., 2001; Tanimoto et al., 1999) to certain cell types, or addition of PPIX or PK11195 in combination with pro-apoptotic agents to other cell types (Bono et al., 1999; Hirsch et al., 1998; Pastorino et al., 1994; Pastorino et al., 1996; Tanimoto et al., 1999) has been shown to potentiate $\Delta\Psi_m$ loss and cell death. Also, PPIX mediates apoptotic death of cells in culture and within brain tumors during photodynamic therapy (Lilge et al., 2000; Noodt et al., 1996). Interestingly, Bcl-2 cannot prevent the pro-apoptotic effects of PK11195 (Fennel et al., 2001; Hirsch et al., 1998) or PPIX (Marchetti et al., 1996). Therefore, Bcl-2 most likely does not prevent apoptosis by physical interaction with the PBR subunit of the PT pore.

Despite being implicated in apoptotic events, clear evidence to show involvement of the PBR in apoptosis modulation has remained elusive. In this chapter, evidence is provided to support a model in which M11L counteracts the pro-apoptotic effects of PBR ligands and physically associates with the PBR subunit of the PT pore. This is the first report of an apoptotic modulator that physically associates with the PBR, thereby identifying a direct role for this PT pore subunit in apoptosis.

## Results

### M11L prevents mitochondrial membrane potential loss

In order to explore the anti-apoptotic properties of M11L more fully, mutant and wild-type forms of the protein were expressed in HeLa cells and their ability to prevent $\Delta\Psi_m$ loss following staurosporine treatment was investigated. EGFP alone, as well as the following chimeric proteins, were expressed with an N-terminal EGFP tag for ease of detection: (1) EGFP-M11L which incorporates full length M11L, (2) EGFP-stop which incorporates a non-targeted, truncated variant of M11L, (3) EGFP-mt, a mitochondrion-targeted form of EGFP directed to mitochondria by just the 25 amino acid mitochondrial targeting sequence of M11L, and (4) EGFP-Bcl-2 which incorporates full-length Bcl-2. The ethyl ester of tetramethylrhodamine (TMRE) was used to detect $\Delta\Psi_m$ loss in this series of experiments as, like $DiOC_6$ (described in Chapter 2), TMRE displays decreased fluorescence intensity as mitochondrial membrane potential diminishes. However, unlike $DiOC_6$, TMRE emits a signal in the orange spectral range that can be distinguished from EGFP fluorescence (Ehrenberg et al., 1988; Farkas et al., 1989).

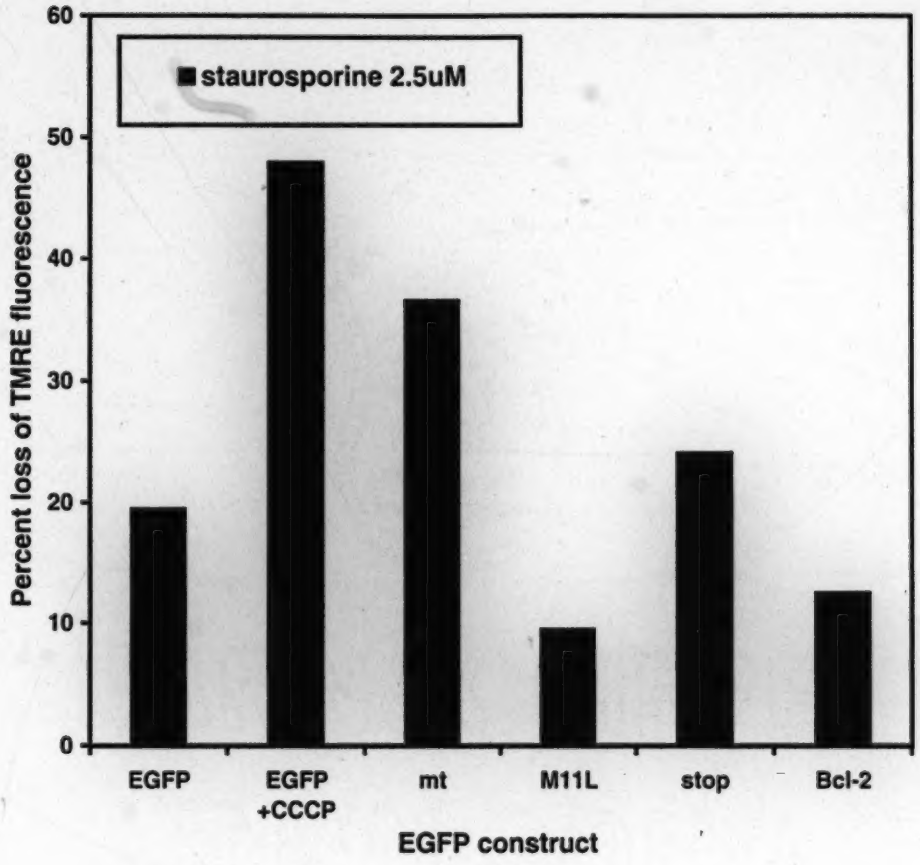


**Figure 4.1 Transiently expressed M11L prevents $\Delta\Psi m$ loss in HeLa cells.** HeLa cells expressing EGFP alone, EGFP targeted to mitochondria (EGFP-mt), or EGFP appended with a truncated form of M11L (EGFP-stop) were not protected from staurosporine-induced loss of TMRE fluorescence. The protonophore CCCP also induced loss of TMRE fluorescence. In contrast, expression of EGFP appended with wild-type M11L (EGFP-M11L) or Bcl-2 (EGFP-Bcl-2) did protect cells from loss of TMRE fluorescence. The results are expressed as the percentage loss of TMRE fluorescence and are the average of two independent experiments.

The percentage of cells expressing EGFP constructs that were also TMRE positive was determined in control and staurosporine treated cells. The reduction in the percentage of TMRE positive cells following staurosporine treatment was then calculated. The average results for two separate experiments are represented graphically in Figure 4.1. As shown in this figure, expression of EGFP alone, EGFP targeted to mitochondria (EGFP-mt) and the non-targeted EGFP-stop truncation mutant, all failed to prevent a loss of TMRE fluorescence. This suggests that these constructs cannot protect mitochondria from undergoing $\Delta\Psi_m$ loss following staurosporine treatment. Control treatment of EGFP-expressing cells with the uncoupler CCCP also induced a marked loss of TMRE fluorescence, indicating that TMRE did provide an accurate measure of $\Delta\Psi_m$ loss. In contrast, expression of the GFP-M11L chimera and the positive control GFP-Bcl-2 construct resulted in TMRE fluorescence being retained in HeLa cells following the same treatment. This indicates that M11L, like Bcl-2, can render mitochondria in HeLa cells resistant to $\Delta\Psi_m$ loss induced by the pro-apoptotic effects of staurosporine. This property is dependent on M11L being correctly localized to mitochondria.

**M11L functionally modulates the PT pore**

Given the mitochondria-based protective effects, one potential mechanism of M11L activity was direct modulation of the PT pore. Therefore, the ability of M11L to counteract the pro-apoptotic effects of PK11195, a PBR ligand that directly targets the PT pore, was investigated. In most cell lines, PK11195 is not pro-apoptotic, but is known to amplify the effects of a number of pro-apoptotic agents (Bono et al., 1999; Hirsch et al., 1998).

The ability of M11L to counteract $\Delta\Psi_m$ loss induced by PK11195 combined with staurosporine was assessed in HeLa cells expressing the EGFP fusion constructs already described. Treatment with PK11195 alone was found

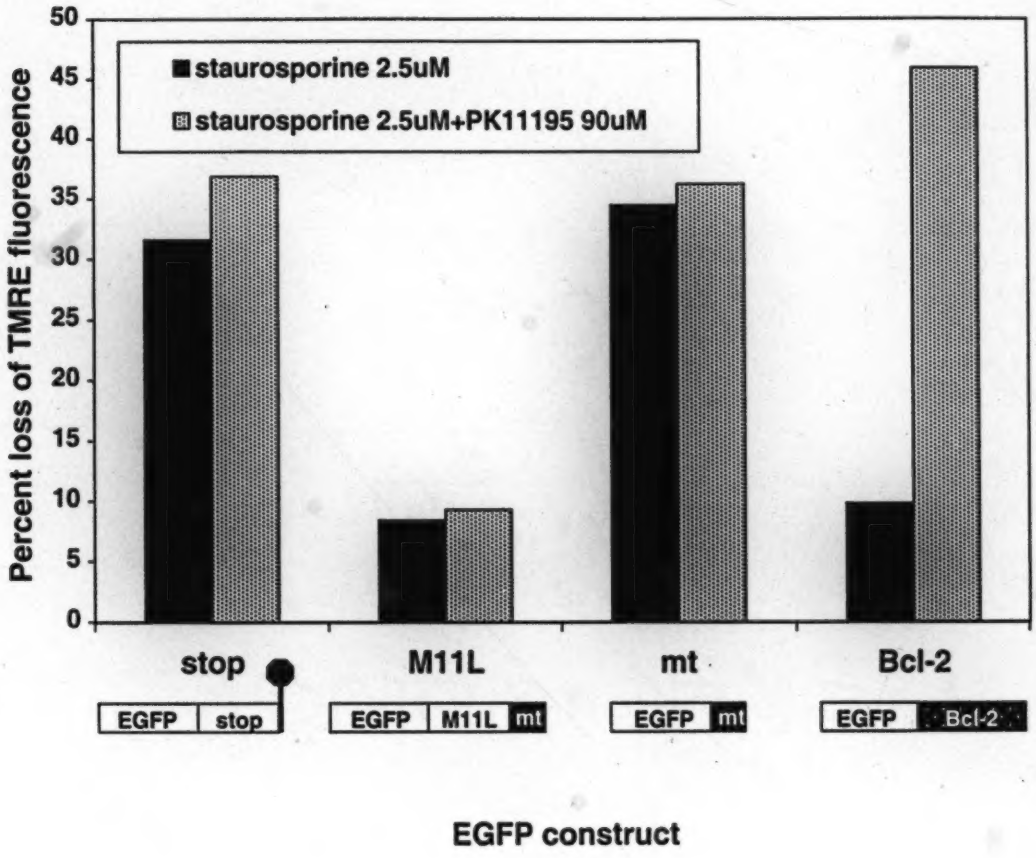


**Figure 4.2 M11L prevents $\Delta\Psi m$ loss in the presence of PT pore ligand PK11195.** When expressed in HeLa cells, EGFP-M11L prevented the loss of TMRE fluorescence in response to staurosporine and staurosporine in combination with PK11195. In contrast, EGFP-mt and EGFP-stop were ineffective in preventing the loss of TMRE fluorescence. EGFP-Bcl-2 was effective against staurosporine alone but not staurosporine in combination with PK11195. The results represent the percentage loss of TMRE fluorescence and are the average of three independent experiments.

not to induce loss of TMRE fluorescence (and by implication $\Delta\Psi_m$ loss) or influence the proportion of EGFP-expressing cells that were TMRE positive (data not shown). Cells treated with PK11195 alone were used to establish the maximal levels of TMRE fluorescence for the calculation of percent loss of TMRE fluorescence. As represented graphically in Figure 4.2, HeLa cells expressing the negative control protein, EGFP-stop or EGFP targeted to mitochondria (EGFP-mt) were not protected from loss of TMRE fluorescence following 2.5 μM staurosporine treatment in the absence or presence of 90 μM PK11195. In marked contrast, cells expressing EGFP-M11L were protected from both treatments. Cells transfected with EGFP-Bcl-2 were protected from staurosporine-mediated apoptosis. However, in keeping with previous studies (Hirsch et al., 1998), the protective properties of EGFP-Bcl-2, which are not thought to occur by direct interaction with the PBR, were clearly counteracted by the combined effects of staurosporine and PK11195. From these results, it can be concluded that M11L is effective in preventing $\Delta\Psi_m$ loss in cells following staurosporine treatment, even in the presence of the PBR ligand PK11195. This ability to counteract the combined effects of PK11195 and staurosporine distinguishes M11L functionally from Bcl-2. These findings provide the first indication that M11L might modulate the PT pore and that this might occur via direct interaction with the PBR.

**M11L function depends on membrane association**

To address a possible link between the mitochondrial localization and function of M11L, it was necessary to compare the anti-apoptotic abilities of wild-type M11L and the C141S and R164G mutants. In these experiments, the functional activities of the EGFP fusion constructs already described and, in addition, the two mutant forms of M11L bearing N-terminal EGFP tags, were analyzed. EGFP chimeras were again expressed in HeLa cells by transient transfection and $\Delta\Psi_m$ loss was

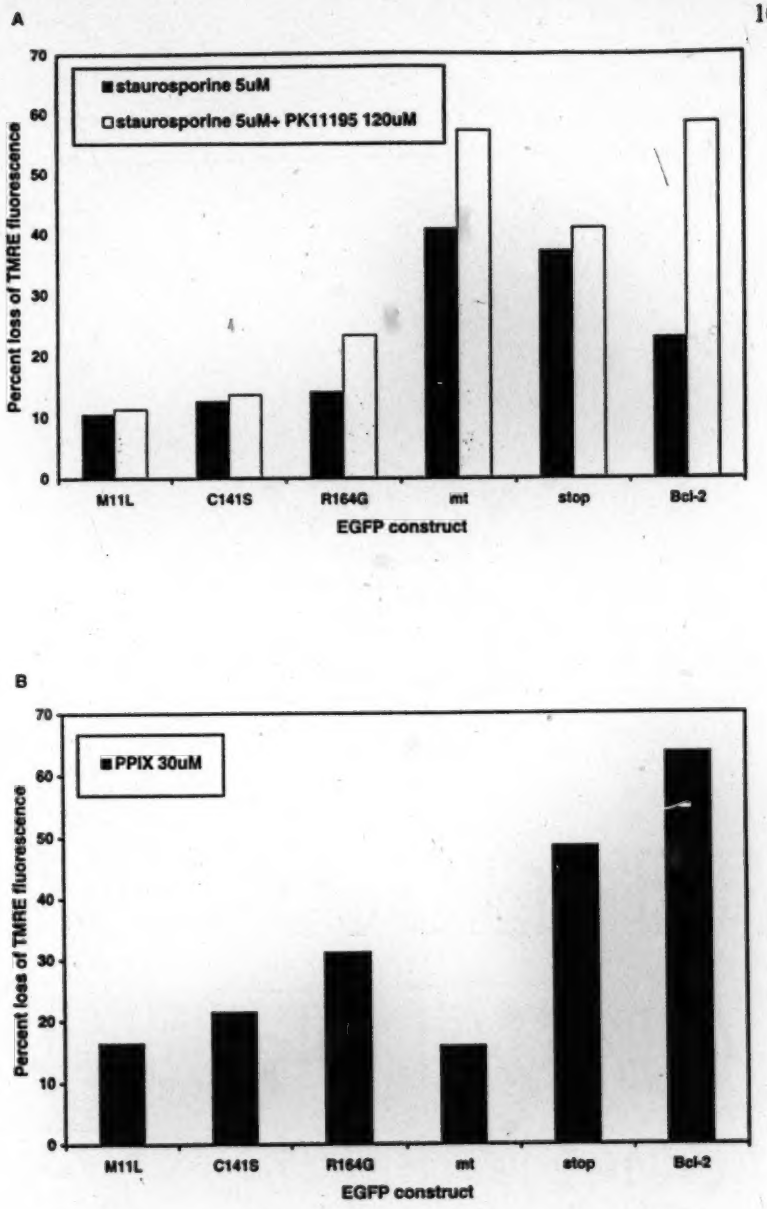


**Figure 4.3 M11L but not the R164G point mutant protects cells from $\Delta\Psi m$ loss potentiated by PT pore ligands.** HeLa cells expressing the EGFP appended with M11L, C141S, R164G, mt, stop or Bcl-2 were treated with (A) staurosporine or staurosporine in combination with PK11195 or (B) PPIX and the percent loss in TMRE fluorescence due to each treatment was determined. The results represent the percent loss of TMRE fluorescence and are the average of two independent experiments.

measured following 5 μM staurosporine treatment in the absence and presence of 120μM PK11195.

As shown in Figure 4.3 (Panel A), EGFP-M11L protected HeLa cells from $\Delta\Psi_m$ loss whereas the negative control EGFP-stop protein and EGFP-mt did not. The properties of the C141S mutant could not be distinguished from those of the wild-type protein. Interestingly, the largely endoplasmic reticulum-localized protein EGFP-R164G was partially active in countering the effects of staurosporine alone, but was less effective in preventing $\Delta\Psi_m$ loss when staurosporine was added in combination with PK11195. EGFP-Bcl-2 was less effective than EGFP-M11L in protecting cells from the higher concentration of staurosporine used in these experiments and the protective effects of EGFP-Bcl-2 were again counteracted by staurosporine in combination with PK11195. From these results, it can be concluded that mitochondria-localized M11L is most effective in counteracting the ability of PK11195 to sensitize cells to staurosporine-mediated $\Delta\Psi_m$ loss.

It was necessary to determine whether M11L could prevent cell death mediated by another apoptotic inducer that interacts directly with the PT pore. The protoporphyrin, PPIX, is an endogenous ligand of the PBR and is known to induce apoptosis when applied directly to cells (Marchetti et al., 1996) or used as the active agent in photodynamic therapy (Lilge et al., 2000). As shown in Figure 4.3 (Panel B), in contrast to the negative control EGFP-stop, EGFP-M11L was effective in preventing PPIX-mediated $\Delta\Psi_m$ loss. EGFP-R164G and EGFP-C141S were less protective than EGFP-M11L and EGFP-Bcl-2 was not at all protective against the concentration of PPIX used. Unexpectedly, the EGFP-mt construct, like EGFP-M11L, was able to prevent $\Delta\Psi_m$ loss following PPIX treatment, indicating a possible role for the 25 amino acid mitochondrial targeting sequence of M11L in interfering with PPIX binding to the PT pore.

In summary, the mitochondria-localized M11L and C141S isoforms of M11L had comparable protective effects against the pro-apoptotic PT pore ligands PK11195 and PPIX. The R164G mutant of M11L, which localizes to different intracellular membranes, had diminished protective effects and the truncated, cytoplasmic isoform of M11L was ineffective against these agents. Therefore in order to be functional, M11L must be membrane associated and preferably localized to mitochondria.

**M11L physically interacts with the mitochondrial permeability transition pore**

Since M11L acts to block apoptotic signals at the mitochondrial control point and prevents loss of $\Delta\Psi_m$ induced by pro-apoptotic PBR ligands, the possibility that M11L could interact directly with protein subunits of the permeability transition pore was worthy of consideration. M11L was over-expressed in Cos-7 cells by infection with a vaccinia virus expression vector (VVM11L). These cells, together with cells infected with control vaccinia virus (VV601), were radiolabelled with $^{35}S$ methionine/cysteine for 12 hours and lysed by resuspension in hypotonic phosphate buffer and dounce homogenization. Samples were divided into duplicates and the hydrophobic crosslinker, Disuccinimidyl suberate (DSS) was added to one sample from each duplicate. The use of a hydrophobic crosslinker was found to be essential for demonstrating M11L binding interactions, perhaps because such interactions require the correct conformation of proteins embedded in membranes. Monomeric and complexed M11L was immunoprecipitated and analyzed by SDS-PAGE and autoradiography.

As shown in Figure 4.4 (Panel A), M11L was only immunoprecipitated from cells infected with the M11L-expressing VVM11L vaccinia virus vector (Lanes 1 and 3) and was not detected in cells infected with control

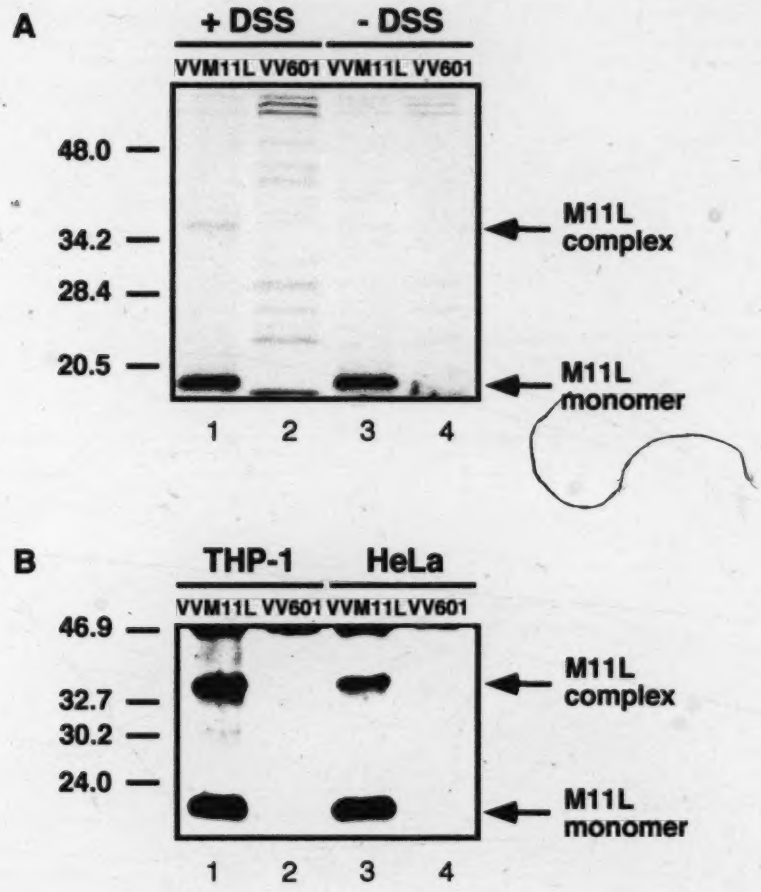


**Figure 4.4 M11L can be crosslinked into a 36 kDa complex.** Cos-7 cells (A) were infected with the M11L-expressing vaccinia virus VVM11L or the control virus VV601 and labeled with 35S methionine/cysteine. Proteins present in hypotonic cell lysates were crosslinked with the hydrophobic crosslinker DSS (+DSS) or left uncrosslinked (-DSS), immunoprecipitated with anti-M11L antibody and analyzed by SDS-PAGE and autoradiography. THP-1 and HeLa cells (B) infected with VVM11L or VV601 were subjected to hypotonic lysis and DSS crosslinking. M11L was immunoprecipitated and detected by SDS-PAGE followed by immunoblot analysis.

vaccinia virus VV601 (Lanes 2 and 4). In addition, when proteins were crosslinked, a complex of approximately 36kDa was observed (Lane 1) and was not observed in the absence of crosslinking (Lane 3).

The presence of M11L in a 36 kDa complex was verified using a similar approach involving infection of THP-1 and HeLa cells with VVM11L or VV601 followed by lysis, crosslinking, immunoprecipitation, SDS-PAGE and immunoblot analysis. As shown in Figure 4.4 (Panel B), monomeric and complexed M11L was detected in THP-1 and HeLa cells infected with VVM11L (Lanes 1 and 3) but was absent from control VV601-infected cells (Lanes 2 and 4). Formation of this crosslinked M11L complex was also observed when experiments were conducted using Cos-7 and HepG2 cells and when myxoma virus (but not an M11L knockout myxoma virus) was used to infect RL-5 rabbit lymphocytes (data not shown).

Formation of a 36kDa complex suggested that the 18kDa M11L protein homodimerizes or associates with a protein of approximately the same molecular weight. As M11L can protect mitochondria from $\Delta\Psi_m$ loss mediated by PBR ligands and the PBR subunit of the PT pore is 18kDa (McEnery et al., 1992), the PBR was a candidate M11L-interacting protein. To address this possibility, we conducted crosslinking experiments as described above, using THP-1 monocytes because these cells express a high concentration of the PBR (Dussossoy et al., 1996). THP-1 monocytes were infected with VVM11L or VV601 and crosslinking was performed with Dithiobis(succinimidylpropionate) (DSP), the cleavable analog of DSS.

Figure 4.5 shows the results following immunoprecipitation of M11L (Panels A and B) or PBR (Panels C and D) from lysates of THP-1 cells infected with VVM11L (Lanes 1 and 2) or control VV601 (Lanes 3 and 4). Samples shown in Lanes 1 to 4 were crosslinked prior to

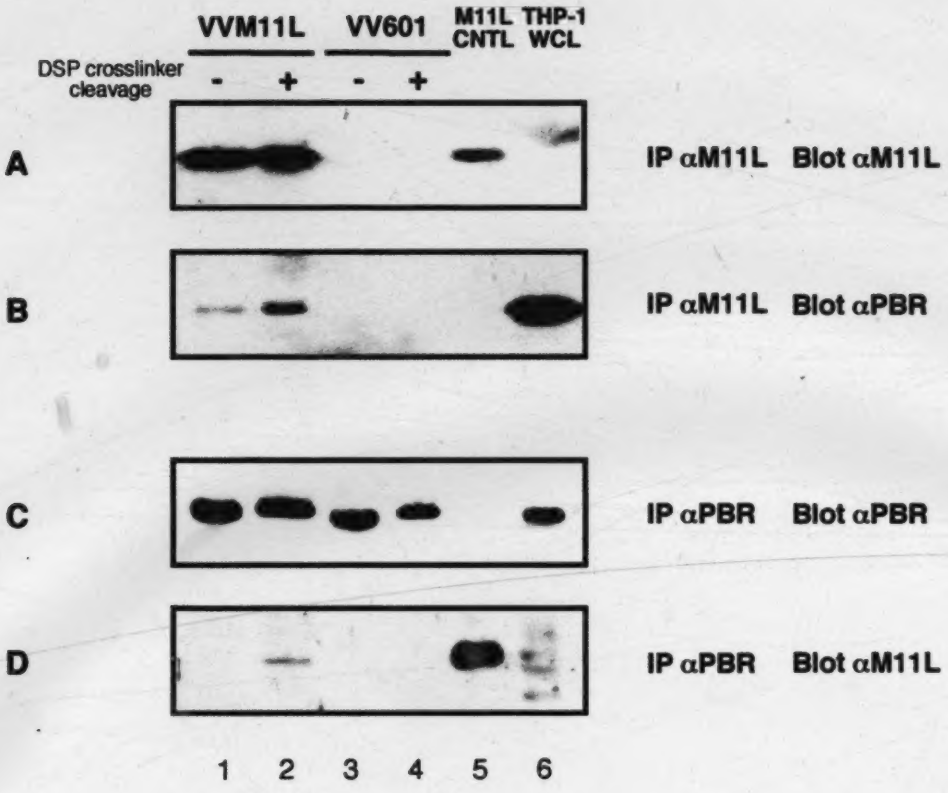


**Figure 4.5 M11L physically interacts with the PBR** THP-1 cells were infected with VVM11L or VV601. Protein within hypotonic cell lysates was crosslinked with DSP and immunoprecipitated with an anti-M11L (Panels A and B) or anti-PBR (Panels C and D) antibody. Samples subjected to crosslinker cleavage (+) or left uncleaved (-) were analyzed by SDS-PAGE and immunoblotting with either an anti-M11L (Panels A and C) or anti-PBR (Panels B and D) antibody. M11L immunoprecipitated from cells infected with VVM11L in the absence of crosslinking or a THP-1 cell whole cell lysate (WCL) were included as controls.

immunoprecipitation and samples in Lanes 2 and 4 were subjected to an additional treatment with reducing agents to cleave crosslinked proteins.

Control samples included were, in Lane 5, M11L immunoprecipitated from VVM11L-infected cells in the absence of crosslinking and, in Lane 6, a THP-1 whole cell lysate. M11L was detected in THP-1 cells infected with VVM11L (Panel A, Lanes 1 and 2) but not in cells infected with VV601 (Panel A, Lanes 3 and 4), as expected. The control samples also confirmed that M11L was immunoprecipitated from VVM11L infected cells (Panel A, Lane 5) and was not detected in the THP-1 whole cell lysate (Panel A, Lane 6), as expected. When the same blot was probed with the anti-PBR antibody, PBR was found to be present in samples immunoprecipitated with the anti-M11L antibody (Panel B, Lanes 1 and 2), indicating that PBR co-precipitated with M11L. Monomeric PBR was detected even in the absence of crosslinker cleavage (Lane 1), indicating that non-crosslinked protein also co-immunoprecipitated with M11L. A higher level of PBR was identified in samples in which the crosslinker had been cleaved prior to SDS-PAGE analysis (Panel B, Lane 2).

The reciprocal experiment, in which crosslinked PBR was immunoprecipitated from THP-1 cells infected with VVM11L and VV601, was also conducted. PBR was correctly detected in immunoprecipitates from infected THP-1 cells (Panel C, Lanes 1-4) and in the THP-1 whole cell lysate (Panel C, Lane 6). PBR was not detected in a control M11L immunoprecipitation in the absence of crosslinking (Panel C, Lane 5). When the same blot was analyzed using an anti-M11L antibody, M11L was identified as having co-precipitated with PBR in VVM11L-infected cells (Panel D, Lane 2). As expected, M11L was also detected in a control M11L immunoprecipitate (Panel D, Lane 5) and was absent from samples infected with VV601 and from the THP-1 whole cell lysate (Panel D, Lanes

3, 4 and 6). We conclude from these crosslinking experiments that M11L interacts directly with the PBR.

**M11L binding to the PBR is biologically relevant**

In order to study the biological importance of the physical association between M11L and the PBR, we investigated the ability of myxoma virus infection to protect cells from staurosporine-mediated apoptosis in the context of different PBR expression levels. PBR-deficient Jurkat T lymphocytes or PBR over-expressing Jurkat-PBR cells (Carayon et al., 1996) were infected with the myxoma virus, vMyxlac, to allow the expression of M11L (data not shown). Cells were treated with 2 μM staurosporine or DMSO solvent as a control and TMRE fluorescence was used to measure $\Delta\Psi_m$ loss. As shown in Figure 4.6, a marked loss of TMRE fluorescence occurred following staurosporine treatment of both Jurkat and Jurkat-PBR cells that had been mock infected, with the presence of PBR only providing a slight protective effect. Myxoma virus infection was found to protect both cell lines from staurosporine-induced $\Delta\Psi_m$ loss. However vMyxlac infection of Jurkat-PBR cells provided a level of protection against $\Delta\Psi_m$ loss that was two fold greater than the protection afforded control Jurkat cells. These results therefore suggest that M11L can synergize with the PBR in infected cells to inhibit apoptosis.

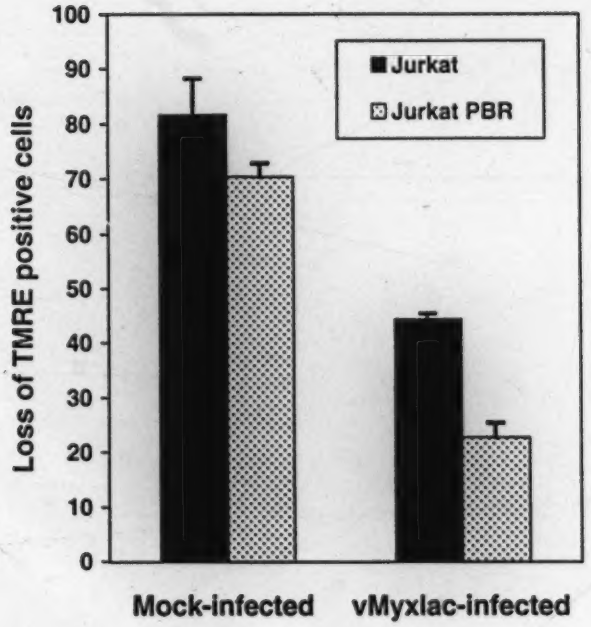


**Figure 4.6 M11L-PBR interactions are biologically relevant.** Mock-infected Jurkat or Jurkat-PBR cells were not protected from $\Delta\Psi_m$ loss induced by staurosporine. vMyxlac infection provided a protective effect that was accentuated by the presence of PBR. The results are the average of three independent experiments and represent the mean TMRE fluorescence + /- SD

**M11L is required to prevent apoptosis during infection of primary rabbit monocytes**

A model in which M11L prevents apoptosis by interacting with the PBR can be expected to have important implications for the *in vivo* situation of myxoma virus infection of rabbits. The PBR is expressed to high levels in cells of the monocyte lineage (Carayon et al., 1996; Dussossoy et al., 1996) and, in addition, is thought to contribute anti-apoptotic (Carayon et al., 1996) but pro-inflammatory properties (Klegeris et al., 2000; Torres et al., 2000). Indeed, as a whole, apoptosis is normally an immunologically silent event that does not elicit an inflammatory response *in vivo* (Savill, 1997). This generalization suggests a contradiction regarding the observation that infection of rabbits with the M11L knockout virus causes an inflammatory disease phenotype, when this same knockout virus induces apoptosis in a cultured rabbit cell line (and presumably also in infected rabbits) and therefore would not be expected to cause inflammation. However, it has been reported that monocyte apoptosis has the unusual property of promoting inflammation (Hogquist et al., 1991; Savill, 1997). It was therefore pertinent to test whether infection of monocytes with the M11L knockout virus was able to induce apoptosis in primary rabbit monocytes, a situation that could explain the pro-inflammatory phenotype of this virus.

An enriched primary rabbit monocyte cell suspension was prepared from peripheral rabbit blood and infected with specific recombinant myxoma virus strains. After 12 h of infection, apoptotic cells were detected by means of the TUNEL reaction. Table 4.1 shows the average percentage of TUNEL positive cells in two independent experiments following 12 h infection.

**Table 4.1 TUNEL analysis of infected primary monocytes**

| Virus | Percent TUNEL positive cells |
|---|---|
| | |
| Mock | 2.1 |
| vMyxM11L$^-$ | 34.3 |
| vMyxlac | 4.5 |
| vMyxM11L$^R$ | 6.3 |
| vMyxlac | 4.5 |
| vMyxT2$^-$ | 5.9 |

Alternatively, TUNEL analysis was conducted and cells of the monocyte lineage were simultaneously identified by the presence of the CD11b surface marker. Figure 4.7 shows representative results from one of four separate experiments.

These studies reveal that only the M11L knockout virus induced notable levels of TUNEL positive cells. In contrast, control vMyxlac, the M11L revertant virus and other myxoma virus constructs with different targeted gene disruptions did not induce elevated levels of dual positive cells, indicating that only the virus which did not produce M11L was able to induce apoptosis in primary rabbit monocytes. This finding is of interest because the M11L, M-T2 and M-T4 deletion mutants are all able to induce apoptosis when infecting cells of the RL-5 lymphocyte line. However, the M11L knockout virus has a distinct and unique phenotype with respect to inducing apoptosis upon primary rabbit monocyte infection and having a pro-inflammatory effect when infecting rabbits. This result identifies a cell lineage where M11L production is functionally significant in preventing apoptosis during infection. Importantly, this data also allows reconciliation of the seemingly conflicting observations that the M11L

knockout virus induces apoptosis but also produces lesions with extensive infiltrations of inflammatory cells in infected animals.

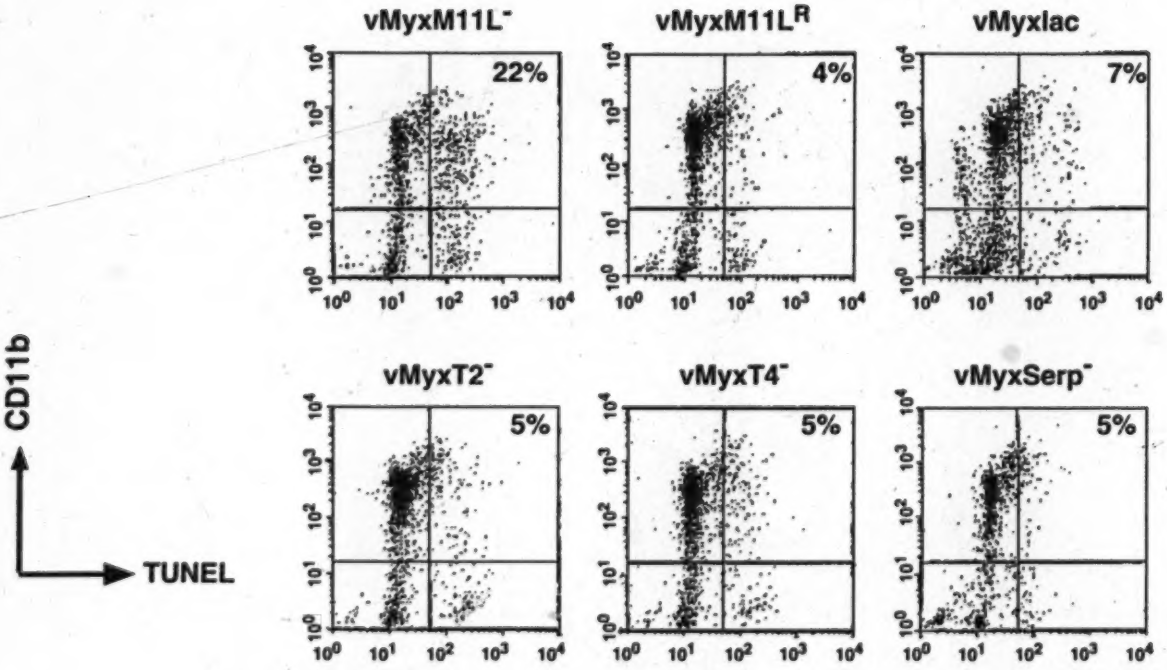


**Figure 4.7 M11L expression during myxoma virus infection prevents monocyte apoptosis.** Primary rabbit monocytes ($CD11b^+$) cells infected with the M11L-expressing recombinant viruses vMyxlac, $vMyxM11L^R$, $vMyxT2^-$, vMyx $T4^-$ and$vMyxSerp^-$ displayed low levels of TUNEL staining. (percentages shown). In contrast, cells infected with the M11L knockout virus $vMyxM11L^-$ exhibited high levels of dual positive staining, indicative of a high level of apoptotic monocytes.

## Discussion

M11L prevents mitochondria from undergoing $\Delta\Psi m$ loss following initiation of an apoptotic signal by staurosporine, a property demonstrated both in Rat2 cells stably expressing M11L (Chapter 2) and in HeLa cells transiently transfected with a GFP-M11L construct (this chapter). TMRE has been successfully used to monitor mitochondrial function in HeLa cells by confocal microscopy (Ehrenberg et al., 1988; Farkas et al., 1989) and in the experiments described in this chapter, was also found to be suitable for flow cytometic analysis, as is the related methyl ester TMRM (Metivier et al., 1998). Although there are pitfalls associated with the use of fluorescent dyes for monitoring mitochondrial function (Bernardi et al., 1999; Poot and Pierce, 1999), the same result was obtained using two different fluorescent probes in the two systems, supporting the conclusion that M11L protects mitochondria from apoptotic changes.

The correct localization of M11L to intracellular membranes and particularly mitochondria appears to be important for its anti-apoptotic function. It is therefore likely that M11L acts as a viral survival effector by preventing amplification of apoptotic cascades that proceed via the mitochondrial pathway. Several other viral anti-apoptotic proteins, notably BHRF-1 encoded by Epstein-Barr virus and vMIA encoded by human cytomegalovirus, have similarly been shown to be important for preservation of mitochondrial function following exposure to apoptosis-inducing agents as outlined in Chapter 1. In the case of M11L and vMIA of cytomegalovirus, this protein expression is also required to sustain viral replication (Goldmacher et al., 1999; Opgenorth et al., 1992).

M11L is a novel protein with no database homologues outside the poxvirus family (Graham et al., 1992). However, it is interesting that

M11L, like the anti-apoptotic Bcl-2 proteins, prevents $\Delta\Psi_m$ loss and mitochondrial transmission of apoptotic signals (Everett et al., 2000; Martinou and Green, 2001; Zamzami and Kroemer, 2001). The mechanistic details underlying the functions of Bcl-2 family members are unclear, but it has been proposed that they exert their modulatory effects by direct physical association with the PT pore. In support of this model, Bcl-2 proteins including Bcl-2, Bcl-$X_L$ and Bax have been shown to physically interact with both the ANT and VDAC subunits of the PT pore (reviewed in (Harris and Thompson, 2000; Martinou and Green, 2001; Zamzami and Kroemer, 2001)). In addition, another novel viral anti-apoptotic protein, vMIA of cytomegalovirus, interacts with the ANT and acts to prevent apoptotic changes downstream of mitochondria (Goldmacher et al., 1999).

EGFP-M11L and other EGFP constructs were characterized with respect to their ability to counteract apoptosis induced by PK11195 and PPIX. Both of these ligands are able to bind PBR in the outer mitochondrial membrane and in the case of PK11195, this occurs independently of VDAC and ANT (Joseph-Liauzun et al., 1997). Mitochondria-localized wild-type M11L and the C141S mutant were found to effectively counteract apoptosis triggered by staurosporine, staurosporine in combination with PK11195, as well as by PPIX. The predominantly endoplasmic reticulum-localized R164G mutant had diminished ability to protect mitochondria from membrane potential loss induced by PPIX or PK11195 in combination with staurosporine. Nevertheless, the R164G variant exhibited some anti-apoptotic activity, particularly against staurosporine. One explanation for this finding is that there may be a endoplasmic reticulum component to staurosporine-mediated death signaling that acts upstream of mitochondria that could be inhibited by both the wild-type and R164G forms of M11L. In addition, there have been reports that a PK11195-binding PBR-like protein is present in the

microsomal fraction of cancer cells (Batra and Iosif, 2000). This raises the possibility that the R164G protein could be binding a PBR-like protein in the endoplasmic reticulum.

The non-targeted M11L truncation mutant failed to protect cells from apoptosis. This parallels observations made with Bcl-2. Bcl-2 is targeted to membranes of the endoplasmic reticulum, outer mitochondrial membrane and perinuclear membrane by means of a C-terminal signal-anchor sequence (Nguyen et al., 1993). Absence of a signal-anchor sequence renders Bcl-2 non-functional, indicating that membrane association is required for function (Nguyen et al., 1994; Tanaka et al., 1993). Bcl-2 constructs specifically targeted by heterologous targeting signals to either mitochondria or the endoplasmic reticulum both possess anti-apoptotic activities in discrete systems (Froesch et al., 1999; Ray et al., 2000; Zhu et al., 1996). The current study therefore reinforces the common requirement of membrane association in order for both M11L and Bcl-2 to be anti-apoptotic.

Nevertheless, in the current study, differences in M11L and Bcl-2 function were observed. M11L displayed greater protective properties than Bcl-2, particularly when high concentrations of staurosporine were used. This may reflect a situation in which M11L, a viral protein, intrinsically has more potent anti-apoptotic properties than Bcl-2 or may reflect the cell death-sensitizing effects resulting from Bcl-2 over expression, both these phenomena having been reported previously (Bellows et al., 2000; Han et al., 1998). Also, in keeping with previous studies, Bcl-2 did not protect cells from the cytotoxic effects of PK11195 in combination with an apoptosis-inducing agent or high concentrations of PPIX (Fennel et al., 2001; Hirsch et al., 1998; Marchetti et al., 1996). In contrast, M11L was protective under these conditions. Bcl-2 has been shown to physically

interact with the VDAC and ANT components of the PT pore, but is not thought to bind directly to the PBR. The ability of PK11195 and PPIX to overcome the anti-apoptotic effects of Bcl-2 can be rationalized if PK11195 binding to the PBR perturbs the entire pore structure and prevents Bcl-2 from binding the other subunits, an effect that is only manifest when another apoptotic stimulus is applied. The observed difference in the properties of M11L and Bcl-2 supports the idea that M11L directly counteracts the pro-apoptotic effects of PBR ligands by inhibiting their binding to the PBR or stabilizing the PBR within the pore structure.

Another interesting observation was that the hydrophobic mitochondrial targeting sequence, mt, might itself possess anti-apoptotic activity, particularly against PPIX, although it was not effective against PK11195 and staurosporine. This may relate to the fact that PK11195 and PPIX have different binding sites on the PBR. This was shown using an antibody directed against an epitope in the extreme C-terminal region of PBR (Dussossoy et al., 1996) which is orientated towards the cytoplasm (Jung et al., 1987). Specifically, this antibody was able to block PK11195 but not PPIX binding, indicating that PK11195, but not PPIX, binds the C-terminal, cytoplasmic domain of PBR. The data described in this Chapter supports the idea that the hydrophobic targeting signal of M11L may interact with PBR in a region overlapping the PPIX binding site. In contrast, the full-length M11L protein may be required to prevent binding of PK11195 to the cytoplasmic C-terminal region of PBR.

The current study reveals that M11L not only protects mitochondria from $\Delta\Psi_m$ loss induced by PBR ligands, but also physically associates with the PBR. This represents the first demonstration of a direct physical interaction between the PBR and an anti-apoptotic protein able to modulate the mitochondrial cell death control point. It was shown previously that vMyxlac infection of the RL-5 rabbit lymphocyte cell line

provides protection against staurosporine-mediated apoptosis (Chapter 2). Interestingly, a similar effect was seen with infected Jurkat T lymphocytes and was accentuated in the presence of elevated levels of PBR. This implies that the physical association of M11L and the PBR is important in infected cells and that the protective effects of these two proteins might be synergistic.

During characterization of the M11L knockout virus, the apparently contradictory observation was made that the loss of M11L function results in the induction of both apoptosis and a massive inflammatory response in infected rabbits. Apoptosis of cells of the monocyte/macrophage lineage is known to have an unusual pro-inflammatory effect. The ability of the M11L knockout virus, as well as various other deletion mutant viruses, to induce apoptosis during infection of primary rabbit monocytes was therefore tested. Whereas a number of these myxoma virus deletion mutants induce apoptosis in the RL-5 lymphocyte cell line, only the M11L knockout virus elicited an apoptotic response in primary rabbit monocytes.

Elevated levels of apoptosis in tissues infected by the knockout virus would not be anticipated in association with signs of an increased inflammatory response except in the unusual situation of apoptosis in cells of the monocyte/macrophage lineage. This pro-inflammatory effect could be attributed to a variety of causes. Monocyte apoptosis has been shown to result in the processing and release of the inflammatory cytokine IL-1β (Hogquist et al., 1991). In addition, monocyte-derived macrophages are responsible for limiting the inflammatory effects of activated neutrophils and other granulocytes. Hence, depletion of the monocyte population could impair the normal regulatory processes of the immune system, which are designed to contain the potentially dangerous effects of uncontrolled inflammation (Savill, 1997).

The ability of M11L to bind to the PBR is also intriguing in the context of monocyte infection and inflammation. Despite having anti-apoptotic properties, the PBR is thought to potentiate inflammatory responses, an effect counteracted by the PBR ligand PK11195 (Klegeris et al., 2000; Torres et al., 2000). Within hematopoeitic lineages, PBR is present in highest concentration in monocytes (Carayon et al., 1996). It is therefore possible that, in monocytes, the M11L-PBR interaction could contribute to the prevention inflammation and apoptosis. Therefore expression of M11L might be particularly important in allowing myxoma virus to productively infect cells of the monocyte/macrophage lineage. This observation is interesting in view of the long recognized importance of macrophages of the reticulo-endothelial system in the development of myxomatosis (Ahlstrom, 1940).

## Materials and Methods

### Cell lines and culture conditions

The Cos-7 monkey kidney, HeLa human cervical carcinoma, THP-1 human monocyte and Jurkat T lymphocyte (clone E6) cell lines were obtained from ATCC. Cos-7 cells were cultured in DMEM (Gibco BRL) and the other cell lines were cultured in RPMI medium (Gibco BRL). All media were supplemented with 10% FBS, 200 U/ml penicillin and 200 µg/ml streptomycin. THP-1 medium also contained 0.1 mM 2-mercaptoethanol. Jurkat-PBR cells (Carayon et al., 1996) (kindly supplied by Dr. P. Casellas, Sanofi Research) were grown in RPMI medium additionally supplemented with 0.1 mM 2-mercaptoethanol and 0.6 mg/ml G418.

### Measurement of mitochondrial membrane potential

HeLa cells were transfected with 4 µg of each of the plasmids pEGFP, pEGFP-M11L, pEGFP-mt and pEGFP-stop using the Lipofectin Plus reagent and Optimem medium (Gibco BRL) according to the manufacturer's specifications. As a positive control, the Bcl-2 coding sequence (kindly provided by Dr. S. Farrow) was cloned as a *Bam*HI-*Xba*I fragment into pEGFP-C1 to allow parallel expression of an EGFP-Bcl-2 chimeric protein. Induction of apoptosis was commenced 20 h post-transfection. Staurosporine was added at a concentration of 2.5 µM for 4 h and control samples were treated with DMSO alone. Measurement of changes in mitochondrial membrane potential in this system was conducted using the ethyl ester of tetramethylrhodamine (TMRE, Molecular Probes) as the accumulation of this dye in the mitochondria of HeLa cells has been shown to correlate directly with the degree of the

negative mitochondrial membrane potential. In addition, this dye displays no appreciable non-specific binding, self quenching or cellular toxicity (Ehrenberg et al., 1988; Farkas et al., 1989). TMRE was added to cells 30 min before harvesting at a final concentration of 0.1 μM. In order to ensure correct detection of $\Delta\Psi m$ loss by TMRE, the protonophore carbonyl cyanide m-chlorophenyl hydrazone (CCCP, Molecular Probes) was added to a control sample. Flow cytometric analysis to detect green fluorescence due to GFP and orange fluorescence due to TMRE was conducted using a Becton-Dickinson FACSCalibur instrument. Green EGFP fluorescence was collected with a 530/30 nm bandpass filter and orange fluorescence from TMRE was collected with a 585/42 nm bandpass filter. A total of 10 000 cells were analyzed per sample with fluorescence signals at logarithmic scale. For each sample, only cells with detectable EGFP fluorescence were considered and the percentage of EGPF-expressing cells that were also TMRE positive was calculated. The percentage loss of dual positive cells following apoptosis induction relative to the control treatment was then calculated in each case.

In order to compare the properties of wild-type M11L with the mutant forms of the protein in the context of PT pore modulation, HeLa cells ($5x10^5$) were transfected with the following EGFP constructs: pEGFP-M11L, pEGFP-C141S, pEGFP-R164G, pEGFP-mt, pEGFP-stop and pEGFP-Bcl-2. Lipofectamine 2000 (Gibco BRL) was used for transfection according to the manufacturer's recommendations with the specific quantities of 1 μg of DNA and 1 μl liposome reagent was used for each transfection. After 24 h, transfected cells were split into replicate wells and the cells were subjected to apoptosis induction. The first treatment involved incubation of cells with staurosporine or staurosporine together with the PBR ligand PK11195 (Sigma) for 4 h. Concentrations of 2.5 or 5 μM staurosporine were used with 90 or 120 μM PK11195 respectively. Replicate cell samples were also treated with PK11195 for control

purposes and all samples were adjusted to contain the same amount of DMSO solvent. The second treatment involved incubation of cells with another PBR ligand, protoporphyrin IX (PPIX, Sigma), at a final concentration of 30 μM for 1.5 h. Control cells were incubated with ethanol solvent alone. During the last 30 min of treatment, the cells were stained with TMRE and analyzed by flow cytometry as described in the previous section.

**Crosslinking assays**

Cos-7 cells ($5x10^6$) were infected at a MOI of 10 with a recombinant vaccinia virus that over-expresses M11L (VVM11L)(Graham et al., 1992) or control vaccinia virus (VV601). Cells were then replenished with DMEM that lacked serum and contained $^{35}S$-labelled cysteine and methionine at a final concentration of 0.1 mCi/ml. Twelve hours post-infection, the cells were harvested and each sample was resuspended in 1 ml cold hypotonic 100 mM potassium phosphate buffer pH 7.4. Samples were subjected to 35 strokes in a dounce homogenizer and then divided into two aliquots. A DMSO solution of the hydrophobic, non-cleavable crosslinker Disuccinimidyl suberate (DSS, Pierce) was added to one aliquot of each sample to a final concentration of 1.25 mM. Samples were incubated at 4°C on a nutator for 2 h, then both monomeric and complexed M11L was immunoprecipitated using a polyclonal rabbit anti-M11L antibody as described in Chapter 2 and identified by SDS-PAGE and autoradiography. Alternatively non-radiolabelled samples were obtained following infection of THP-1 or HeLa cells for immunoblot analysis.

**Crosslinking and co-immunoprecipitation assays**

THP-1 cells ($2x10^6$) were infected at a MOI of 10 with VVM11L or VV601. Twelve hours post-infection, cells were subjected to hypotonic lysis,

dounce homogenization and crosslinking as described above, except in this case, the hydrophobic, cleavable analog of DSS, Dithiobis(succinimidylpropionate) (DSP, Pierce) was used at a final concentration of 0.125 mM. Samples were lysed in NP40/deoxycholate lysis buffer (50 mM Tris-HCl pH8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 2 mM EDTA and 1 mM PMSF) and divided into duplicates. Duplicate samples were immunoprecipitated with a polyclonal rabbit anti-M11L antibody and crosslinked complexes in one sample from each duplicate were cleaved into their monomeric components by incubation at 65°C in SDS-PAGE loading buffer containing 100 mM dithiothreitol and 0.2% 2-mercaptoethanol. In parallel, the same procedure was conducted on samples immunoprecipitated with a polyclonal rabbit anti-PBR antibody(Dussossoy et al., 1996) (kindly supplied by Dr. P. Casellas, Sanofi Recherche). PBR and M11L proteins were detected using SDS-PAGE and immunoblot analysis.

**Measurement of $\Delta\Psi_m$ in infected Jurkat cells**

Jurkat or Jurkat-PBR cells ($5x10^5$) were mock-infected or infected with the M11L-expressing myxoma virus, vMyxlac (Opgenorth et al., 1992), at a MOI of 20. Ten hours post-infection, 2 mM staurosporine or an equivalent volume of DMSO was added to the cells. Following incubation for 3h, 50 nM TMRE was added to the medium for 30 min. TMRE fluorescence was measured by flow cytometry as described above.

**Isolation, infection and analysis of primary rabbit monocytes**

For each experiment, heparinized blood was obtained by cardiac puncture from a healthy New Zealand White laboratory rabbit and subjected to Ficoll-Paque (Pharmacia) density gradient separation. The buffy coat containing white blood cells was collected and cells were cultured in RPMI medium containing 20% FBS for 4 h and the monocyte population was enriched as a result of adherence to the plastic culture dish.

The adherent cells were detached from the plastic support using warm 1xSSC (150 mM sodium chloride, 15 mM sodium citrate, pH 7.5) and infected with different recombinant myxoma virus isolates at a MOI of 10. The viruses used included control vMyxlac, a which produces M11L, vMyxM11L$^-$ which fails to produce M11L owing to a targeted gene disruption (Opgenorth et al., 1992) and vMyxM11L$^R$, a revertant virus in which the gene disruption has been repaired (Macen et al., 1996). Other myxoma virus constructs with targeted gene disruptions were incorporated in this study for purposes of comparison and included the M-T2 (Upton et al., 1991), M-T4 (Barry et al., 1997) and Serp-1 (Macen et al., 1993) recombinant myxoma viruses. Typically, infection rates exceeded 70% as verified by staining with X-gal (Opgenorth et al., 1992).

The infected cells were cultured in medium containing 20% FBS for 12 h, fixed in 2% paraformaldehyde/PBS and apoptotic cells were detected using the TUNEL reaction. The monocyte population in each sample was identified by indirect immunofluorescence by first incubating with a mouse anti-rabbit CD 11b antibody (Spring Valley) at a dilution of 1:50 at room temperature for 20 min followed by incubation with a phycoerythrin (PE)-conjugated F(ab)'2 goat anti-mouse secondary antibody (DAKO) at a dilution of 1:20 at room temperature for 20 min. Data were acquired using a Beckton-Dickinson FACScan flow cytometer using the same settings described for the FASCalibur instrument. The fluorescein-dUTP signal

from TUNEL positive cells and PE fluorescence from CD11b positive cells was analyzed for the gated cell population.

# Chapter 5

## Future Directions

Viral proteins are exquisitely sensitive with regards to the cellular molecules and therefore the pathways that they modulate. For this reason, the study of virus-directed processes that occur within cells is interesting from a virology point of view, and can also provide valuable insights into cellular function. In addition, viral proteins can not only serve as sensitive tools to dissect cellular mechanisms but also potentially have uses as biological theraputic agents. M11L is one such protein that continues to be useful for the investigation of apoptosis. The studies described in this thesis suggest further biochemical aspects of M11L function that await exploration and also possible theraputic implications for this protein.

**M11L: Biochemical aspects**

The studies described in this thesis show that M11L is anti-apoptotic. However, only a few apoptotic agents have been tested thus far, and it would be interesting to determine the full extent of the protective effects of M11L and its mutants. An investigation in this area my serve to further delineate differences in forms of M11L that are differently targeted.

During the investigation into the biological relevance on M11L-PBR interactions, infection with M11L-expressing myxoma virus was observed to provide a greater protective effect in PBR-containing cells in comparison to PRB-deficient cells. Nevertheless myxoma virus infection of PBR-deficient cells still did provided a measure of protection in comparison to uninfected cells. This indicates that the anti-apoptotic properties of M11L might be due to interactions with cellular proteins in addition to the PBR.

In order to address the question of whether M11L is able to bind to other cellular proteins in addition to the PBR, an plasmid construct was

generated to allow the expression of M11L appended with an N-terminal Flag tag. Flag tagged M11L was transiently expressed in HEK293 cells, immunoprecipitated with an anti-Flag antibody and co-precipitating proteins were analyzed by microsequencing. Two proteins consistently co-precipitated with M11L, namely the pro-apoptotic Bcl-2 family member, Bak and the structural protein, tubulin (Drs. Karen Colwill and Mike Moran, MDS Proteomics, personal communication). It is therefore pertinent to investigate whether these interactions, particularly with reference to Bak, are also observed during myxoma virus infection as well as in cells stably expressing M11L. This can be achieved by means of crosslinking experiments using infected and Rat2M11L cells. A protocol similar to that used to show the M11L-PBR interaction could be employed.

To explore the functional consequences of M11L expression, it will be of use to generate cell lines that stably express mutant and wild-type forms of M11L. Work is already in progress to generate HeLa cell lines that express these proteins, as apoptotic pathways induced by a wide array of agents are well characterized in this cell line. These cell lines can be used to show that M11L protects cells from apoptosis involving Bak activation eg triggered by thapsigargin, Fas, UV irradiation and other inducers. These cell lines will also greatly facilitate investigation of whether M11L prevents Bak insertion and/or oligomerization in the mitochondrial outer membrane.

**M11L: Potential uses**

The study of M11L might also have wider relevance in terms of disease pathologies (Constantini et al., 2000; Kroemer and Reed, 2000; Nicholson, 2000). Apoptosis has been strongly implicated in chronic neurodegenerative conditions, notably Alzheimer's disease and Huntington's disease. Apoptosis also plays a role in autoimmune

conditions and transplant rejection that result from activity of cytotoxic T lymphocytes (CTL) and natural killer (NK) cells. M11L has potential to prevent apoptosis of target cells in these disease processes. It would therefore be of interest to study the ability of M11L to prevent the death of neurons and also to counteract CTL and NK cell-mediated death.

Mitochondrial toxicity associated with drug treatments is emerging as a matter of concern. Toxicity can arise, for example, during anti-HIV therapy and has also been associated with aspirin that can cause Reye's syndrome when taken during a prodromal viral illness (Kroemer and Reed, 2000). As M11L acts at the mitochondrial control point of cell death, it would be of interest to explore the role of M11L in preventing drug-mediated toxicity. Reye's syndrome is thought to involve the PBR, a molecule that is implicated in apoptotic signal transduction and the potentiation. M11L interaction with and stabilization of the PBR therefore has implications for the prevention of apoptosis. As described in the previous Chapter, PBR is also implicated in mediating inflammation. Therefore an interaction between M11L and PBR could have implications for preventing inflammation. It is of particular note that preliminary evidence exists to suggest that a short 25 amino acid hydrophobic region of M11L responsible for mitochondrial targeting may, itself, have anti-apoptotic properties against some cell death triggers. Hence, the ability of this peptide, in various carrier formulations, could be tested for ability to prevent apoptosis when delivered directly to cells.

In conclusion, M11L belongs to a new class of viral apoptotic inhibitors that impact the mitochondrial cell death control point. These proteins have the potential to serve as valuable tools for the investigation of apoptotic pathways and possibly, for theraputic intervention. The study of M11L promises to be a fruitful area of research in the future.

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